REMARKS

The application has been objected to as the sequence disclosures have not been complied with. The description of figure 5 has been amended to comply with the rules.

The disclosure has been objected to due to minor informalities. The specification has been amended to correct the informalities including correct arrangement of the specification.

Claims 1-5, 7-11, 14, and 23 have been rejected under 35 U.S.C. §112, first paragraph. The claims have been amended to obviate the examiner's rejection.

Figure 5 discloses four other sequences that share homologies with a sequence of qde-1 protein. In particular, as described at page 18, lines 14-24, the significant homology with the tomato RNA-dependent RNA polymerase RdRP is limited to the aa. region 710-1282. Therefore, a representative number of species has been affectively described, by their sequence homology restricted to the claimed aa. region. Additionally, the species have also been described by other relevant identifying characteristics, namely the RNA-dependent RNA polymerase activity.

The examiner has stated that the specification fails to disclose what domain is necessary and sufficient for the silencing activity. This is not claimed subject matter and thus is not required. Claim 1 refers to a nucleotide sequence coding for a protein having a silencing activity (all the protein) and comprising a RNA-dependent RNA polymerase domain. No claim to a domain having both silencing and RNA-dependent RNA polymerase activities is present.

As to the silencing activity of such groups of proteins enclosed herewith are following documents all of which have publication dates after the instant application:

Encl. 1: Dalmay et al. Cell 101, 543-553, May 26, 2000, that confirms the homology of the tomato RdRP with qde-1 (see Summary and Figure 4B) and also that such gene family has a silencing activity.

Encl. 2: Catalonotto et al. Genes and Development 16, 790-795, 2002, that shows that the presence of small ubiquitous RNAs (that are essentially for silencing activity in all of organisms) depends upon the activity of qde-1, confirming the role of such protein in silencing (see in particular Figure 2 p. 791).

Encl. 3: Makeyev et al. Molecular Cell 10, 1417-1427, December, 2002, that confirms that qde-1 of N. Crassa is an RdRP (see in particular Figure 1, B and C, p. 1418).

Encl. 4: Forrest et al. Nucl. Acid Res. 32, 2123-2128, 2004, that further confirms that qde-1 of N. Crassa is relevant and essential in silencing.

The applicants found for the first time that qde-1 is essential in silencing and has a domain of RdRP as acknowledged by the Examiner on page 9. Such an original finding has been confirmed by many scientists after the patent application filing date. It is thus believed that the written description requirement satisfies the requirement.

As to the enablement requirement, the applicants disagree with the examiner's assertion that the teaching of the specification is limited. Figure 5 discloses other members of the family sharing claimed homologies. Moreover, as to the tomato RdRP, it has been demonstrated that the protein has a silencing activity in plants as disclosed in enclosure 1. Again, it must be

noted that the claim is not directed to the domain itself but to a protein comprising such domain.

The documents provided by the examiner that should demonstrate the unpredictability of a function given a sequence homology are respectfully not pertinent, but refer to a completely different and unrelated protein and biological system. In the instant case, all of documents published further to the filing of the application confirmed such a relationship.

In view of the foregoing, it is believed that the specification, amended claims, and the claims dependent there from are in proper form. The application is now considered to be in condition for allowance, and an early indication of same is earnestly solicited.

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An RNA-Dependent RNA Polymerase Gene in *Arabidopsis* Is Required for Posttranscriptional Gene Silencing Mediated by a Transgene but Not by a Virus

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Summary

Posttranscriptional gene silencing is a defense mechanism in plants that is similar to quelling in fungi and RNA interference in animals. Here, we describe four genetic loci that are required for posttranscriptional gene silencing in *Arabidopsis*. One of these, *SDE1*, is a plant homolog of *QDE-1* in *Neurospora crassa* that encodes an RNA-dependent RNA polymerase. The *sde1* mutation was specific for posttranscriptional gene silencing induced by transgenes rather than by viruses. We propose that the role of SDE1 is to synthesize a double-stranded RNA initiator of posttranscriptional gene silencing. According to this idea, when a virus induces posttranscriptional gene silencing, the virus-encoded RNA polymerase would produce the double-stranded RNA and *SDE1* would be redundant.

Introduction

Posttranscriptional gene silencing (PTGS) in plants, quelling in fungi, and RNA interference in animals are responses to various types of foreign nucleic acid including viruses, transposons, transgenes, and double-stranded (ds)RNA (Vaucheret et al., 1998; Sharp, 1999). These processes represent natural systems of defense against viruses (Ratcliff et al., 1997, 1999) and transposons (Ketting et al., 1999) that were first discovered from experiments with transgenes (Napoli et al., 1990; van der Krol et al., 1990), virus vectors (Ruiz et al., 1998), or injected dsRNAs (Fire et al., 1998) in which the foreign nucleic acids were based on endogenous genes. The foreign nucleic acids caused the defense system to be targeted against these endogenous RNAs so that the organism exhibits a phenocopy of loss-of-function mutations in the corresponding genes.

In *Neurospora crassa* and *Caenorhabditis elegans*, there is compelling evidence that the quelling and RNA interference mechanisms are related because both are abolished by mutations in homologs of a Werner's disease syndrome gene (Cogoni and Macino, 1999b; Ketting et al., 1999), an eIF2C translation factor gene (Tabara et al., 1999; Catalanotto et al., 2000), and an RNA-dependent RNA polymerase (RdRP) gene (Cogoni and Macino, 1999a; Smardon et al., 2000). In due course, this list will be extended by characterization of other loss-of-PTGS or enhanced PTGS mutants in these organisms (Cogoni and Macino, 1997; Ketting et al., 1999; Tabara et al., 1999) and *Arabidopsis* (Dehio and Schell, 1994; Elmayan et al., 1998).

Biochemical analysis has also contributed to understanding of PTGS through the identification of short (25 nucleotide) RNAs that are invariably associated with PTGS in plants. These short RNAs of both sense and antisense polarity correspond to the target of PTGS (Hamilton and Baulcombe, 1999). Similar short RNA species have been associated with RNA interference in *Drosophila melanogaster*. In in vitro extracts, they are produced by processing of larger dsRNAs (Zamore et al., 2000) and provide sequence specificity to a system of RNA degradation (Hammond et al., 2000).

To shed more light on the mechanism of PTGS, we carried out a mutation analysis of PTGS in *Arabidopsis* carrying two transgenes (Dalmay et al., 2000). One of these transgenes encodes a potato virus X:GFP vector (PVX:GFP) and is responsible for initiation of PTGS. The second transgene encodes a green fluorescent protein (GFP) reporter of silencing. In the absence of the *PVX:GFP* transgene, this reporter gene was expressed at a high level and the *Arabidopsis* plants were green fluorescent under UV light. However, in the presence of the *PVX:GFP* transgene, there was PTGS of the GFP reporter. This system was designed to combine elements of transgene- and virus-induced PTGS.

We report here that at least four genetic loci are required for PTGS in *Arabidopsis*. Mutant, silencing *defective* (*sde*) plants differ from wild type in that they accumulate high levels of *GFP* and *PVX:GFP* RNAs and low levels of the 25 nt RNAs associated with PTGS. We show that one of these mutant loci (*sde1*) encodes an RdRP-related protein. This finding confirms the similarity of PTGS in plants with gene silencing phenomena in other organisms because the SDE1 protein is similar to QDE-1 of *N. crassa* and to EGO-1 of *C. elegans*, which are required for quelling and RNAi, respectively (Cogoni and Macino, 1999a; Smardon et al., 2000). The SDE1 protein is required for transgene silencing but not for virus-induced PTGS and, based on that finding, we propose that the role of SDE1 is to produce a dsRNA activator of PTGS. According to this idea, SDE1 is not required for virus-induced PTGS because the virus-encoded RdRP produces dsRNA as an intermediate in the replication cycle.

Results

Mutation Analysis of PTGS in *Arabidopsis*

To identify genes required for PTGS, we carried out fast neutron mutagenesis of an *Arabidopsis* line [GFP142xAmp243] (Dalmay et al., 2000). This line is derived from the F2 progeny of a cross between parental lines GFP142 and Amp243 carrying 35S-*GFP* and 35S-*PVX:GFP* transgenes, respectively. We refer here to [GFP142xAmp243] as "GxA" and the Amp243 and GFP142 parental lines as "A" and "G", respectively.

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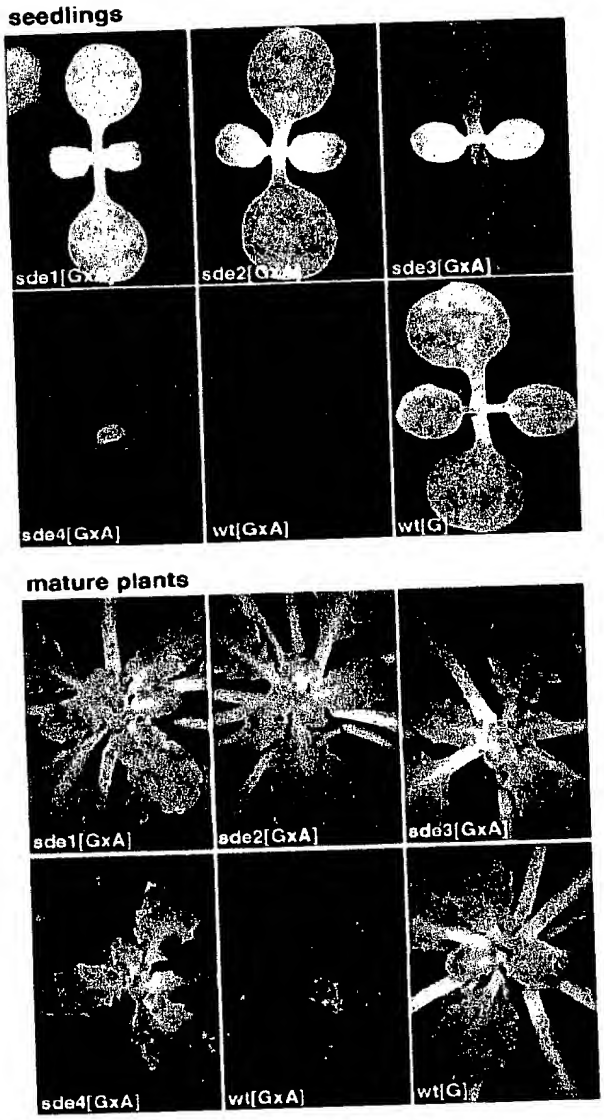

Figure 1. GFP Fluorescence in Progenitor and *sde* Mutant Plants

The images were produced under UV light in a dissecting microscope and the red fluorescence is due to chlorophyll. GFP fluorescence appears green or yellowish-green in different tissues. The images in the top and bottom six panels were taken one and three weeks after germination, respectively. The images are of wild type (wt), G, and GxA plants and of GxA plants carrying *sde1*, *sde2*, *sde3*, and *sde4* mutations as indicated. The distorted leaf phenotype of the *sde1* and *sde2* mutants is not evident in these images. Note the red fluorescent petioles on the lower, older leaves of the *sde3* mutants of GxA.

The GxA line carries both transgenes in the homozygous condition and exhibits strong PTGS, manifested as the almost complete absence of *GFP* RNA (Dalmay et al., 2000). Under UV light, these plants exhibit red fluorescence due to chlorophyll rather than the green fluorescence of GFP (Figure 1). The presence of both transgenes is essential for the strong PTGS phenotype of this line. When present alone, the 35S-*PVX:GFP* from line A is able to mediate only weak PTGS and the 35S-*GFP* transgene from line G is not silenced (Dalmay et al., 2000). We anticipated that loss of PTGS in *sde* mutants of GxA would prevent the degradation of *GFP* mRNA and that the plants would be as green fluorescent as the parental line G (Figure 1).

From a total of 150,000 M2 plants of 50 families, we identified 64 plants that were candidate *sde* mutants of GxA. The representative GFP phenotypes of these plants are shown in Figure 1 with separate panels for seedlings and mature plants. Of the 64 candidates, 51 were similar to wild-type line G: these plants exhibited the GFP fluorescence in all tissues and throughout development. We refer to this phenotype as "full green" (Figure 1, sde1[GxA] and sde2[GxA]). A secondary phenotype on some of these full green plants was slight distortion of the leaf margins. A further 12 mutant plants showed delayed loss of PTGS (Figure 1, sde3[GxA]). The hypocotyl and cotyledons were red fluorescent, as in the wild-type plants, but the true leaves were green fluorescent, indicating that PTGS was lost. These plants remained green fluorescent throughout development, although the fluorescence was slightly less intense than in the full green plants and the leaves did not show any distortion. A third phenotype in a single plant was transient loss of PTGS (Figure 1, sde4[GxA]). The young, newly emerging leaves of this "transient green" plant were green fluorescent but the older, mature leaves were red fluorescent, as in the wild type (Figure 1, wt[GxA]). Emerging leaves on these plants had small spots of red fluorescence that eventually converged so that entire leaves were covered.

The PTGS in GxA depends on replication of the *PVX:GFP* RNA encoded by the 35S-*PVX:GFP* transgene (Dalmay et al., 2000), and loss of PTGS could be due to mutation either of the transgene, of host genes required for virus replication, or of genes required for silencing. To differentiate these possibilities, we exploited our previous observation that weak PTGS in line A permits some accumulation of PVX:GFP, whereas the enhanced PTGS in GxA does not (Dalmay et al., 2000). We reasoned that mutation of a host gene required for PTGS would allow accumulation of infectious PVX:GFP in GxA. However, if the mutation was in the 35S-*PVX:GFP* transgene or in a host gene required for virus replication, there would be no accumulation of PVX:GFP.

To assay for infectious PVX:GFP we inoculated *Nicotiana benthamiana*, a good host of PVX, with extracts of the green fluorescent GxA mutants. The presence of virus was diagnosed by symptoms of PVX and by expression of viral GFP in the inoculated and systemically infected leaves. This test showed that 12 out of the 64 *Arabidopsis* lines did not contain PVX:GFP, indicating that they harbored mutations in the 35S-*PVX:GFP* transgene or in a host gene required for virus replication. These mutants were not used further in our analysis of PTGS. The remaining lines did contain PVX:GFP and were candidate *sde* mutations. From a total of 190 crosses, we classified the mutant loci in four complementation groups: two with a full green phenotype (*sde1* and *sde2*, 4 and 3 alleles of each), one with delayed green phenotypes (*sde3*, 3 alleles), and one with the

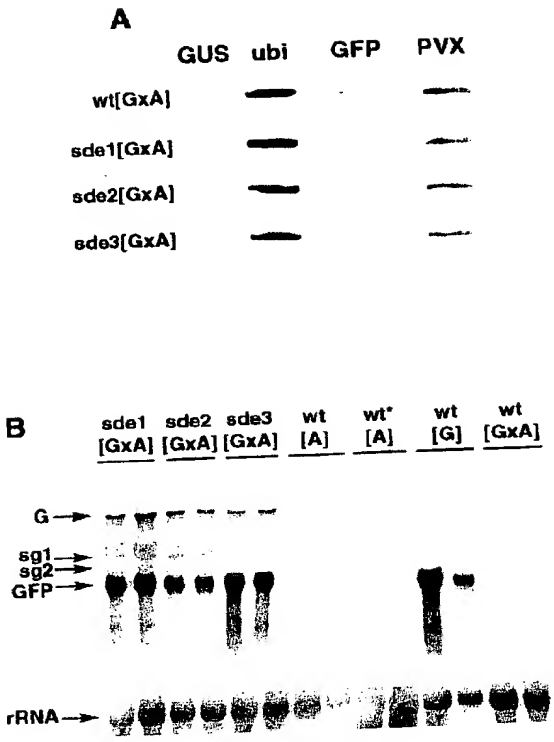

Figure 2. Nuclear Run-Off and Northern Blot Analysis of Transgene Expression

(A) GUS, *ubiquitin* (ubi), *GFP*, and PVX cDNAs were fixed onto nitrocellulose membranes that were hybridized with the ^{32}P-labeled in vitro transcripts of isolated nuclei from *sde* or wild-type (wt) [GxA] lines.

(B) RNA was isolated from young leaves of wild-type (wt) A, G, GxA, and from *sde* mutants of the [GxA] plants. Northern blot analysis was carried out using ^{32}P-labeled probes corresponding to the full-length *GFP* RNA sequence (top) or rRNA (bottom). The RNA species detected were the genomic (G) and subgenomic (sg) length RNAs of PVX:GFP: *GFP* mRNA (GFP) and rRNA. The lane marked with an asterisk (*) contains five times more RNA than the others to show the subgenomic RNAs in the wt A plant.

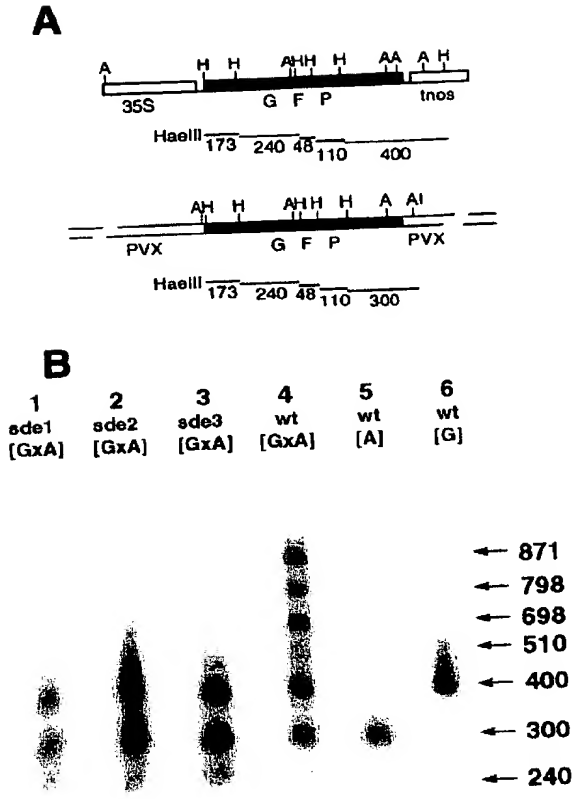

Figure 3. Transgene Methylation in Wild-Type and *sde* GxA Plants

(A) Structure of the *GFP* transgene in 35S-*GFP* and 35S-*PVX:GFP*. Features shown are the 35S promoter (35S) *GFP* coding sequence (black box, *GFP*), nopaline synthase terminator (tnos), and the PVX sequence flanking *GFP* in 35S-*PVX:GFP*. The restriction sites for HaeIII (H), and the lengths (in base pairs) of the expected digestion products are shown.

(B) Southern blot analysis with DNA from leaves of *sde1*[GxA] (lane 1), *sde2*[GxA] (lane 2), *sde3*[GxA] (lane 3), wild type (wt) [GxA] (lane 4), wt A (lane 5), and wt G (lane 6) plants using a full-length *GFP* sequence probe. The numbers indicate the estimated length (in base pairs) of the hybridizing DNA fragments.

transient green phenotype (*sde4*, 1 allele). All *sde1* and *sde2* lines with the full green phenotype exhibited the slight distortion of the leaf margins. In our further analysis of the *SDE* loci, we decided to focus initially on *SDE1*, *SDE2*, and *SDE3* because mutations at these loci caused the most complete loss of PTGS.

In further tests of transgene RNA levels and transcription in the wild-type and mutant lines, the results confirmed a PTGS basis for the *sde* mutations. Thus, in nuclear run-off analysis (Figure 2A), the transgene transcription in *sde1*, *sde2*, or *sde3* lines was the same as in the wild-type GxA, whereas the steady state levels of the *GFP* and *PVX:GFP* RNAs were much higher (Figure 2B). The *GFP* RNA from the 35S-*GFP* transgene was as abundant in the mutant lines as in the nonsilenced G line (Figure 2B). The level of *PVX:GFP* RNA from the 35S-*PVX:GFP* transgene was higher than in the parental A line (Figure 2B), in which there was weak PTGS. Based on these data, we consider it likely that the *SDE* loci encode factors, most likely proteins, required for PTGS. In the *sde1* and *sde2* lines, in which there was slight distortion of the leaves, the viral RNAs were more abundant than in the *sde3* lines in which the leaves were normal. It is likely that these two phenotypes are related and that leaf distortion is a symptom of PVX:GFP accumulation.

Transgene Methylation in *sde* Mutants

In line GxA, the PTGS is associated with methylation of the 35S-*GFP* and 35S-*PVX:GFP* transgenes (Dalmay et al., 2000). The *GFP* DNA in this line is partially resistant to digestion by the methylation-sensitive restriction enzyme, HaeIII (Figure 3B, lane 4). In contrast, in the parental lines A and G, in which silencing was weak or absent, the *GFP* DNA was completely digested by these enzymes, indicating that it was not methylated (Figure 3B,

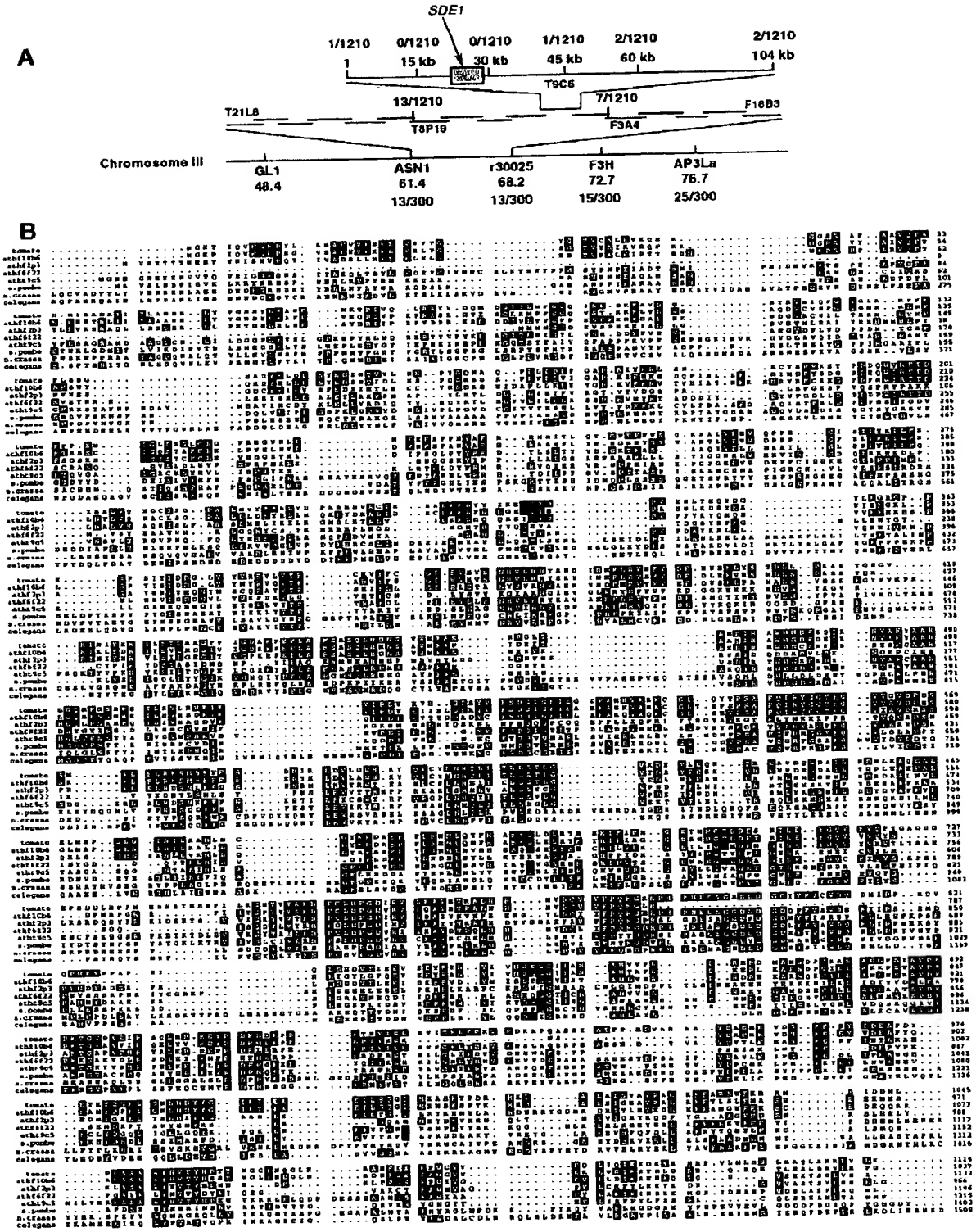

Figure 4. Genetic Map of the *SDE1* Region and Amino Acid Sequence of SDE1

(A) The *sde1* phenotype cosegregated with *GL1* and *F3H* markers on chromosome 3. Twenty-six recombination events were detected between *ASN1* and r30025 among 300 chromosomes examined. There were 13 recombination events between *ASN1* and *SDE1* and between *SDE1* and r30025, indicating that *SDE1* is located between *ASN1* and r30025. A *QDE-1* homologous sequence was identified on BAC T9C5. The BAC clones around T9C5, generated as part of the Arabidopsis Genome Sequencing Project, are indicated as horizontal lines. Close flanking

lanes 5 and 6). The *GFP* DNA from the *sde* mutant lines was also digested to completion, indicating that, associated with the loss of PTGS, the methylation of *GFP* DNA was prevented (Figure 3B, lanes 1–3).

SDE1 Encodes a Homolog of the *Neurospora* QDE-1 RNA Polymerase

We cloned *SDE1* based on its position in the *Arabidopsis* genome. First, we crossed lines carrying the *sde1-1* allele (C24 ecotype) to a wild-type plant of the Landsberg ecotype. Then, in the F2 generation, we tested plants carrying both the 35S-*GFP* and 35S-*PVX:GFP* for cosegregation of the mutant *sde1* phenotype with markers from each of the five *Arabidopsis* chromosomes. From this analysis, we showed that *sde1* is on the bottom arm of chromosome 3 between markers *GL1* (48.4 cM) and *F3H* (72.7 cM) (Figure 4A).

Further tests with chromosome 3 markers narrowed down the *SDE1* locus to an interval of 6.8 cM (between markers *ASN1* and r30025) at position 61.4–68.2 cM (Figure 4A). This interval includes a homolog of a gene in *N. crassa* (*QDE-1*) that is required for quelling (Cogoni and Macino, 1999a). We therefore tested the possibility that *SDE1* is the *Arabidopsis* homolog of *QDE-1* by segregation analysis of close flanking markers and of the DNA sequence. The close flanking markers confirmed that a 45 kb interval spanning the *QDE-1* homolog contained *SDE1* on BAC T9C5 (Figure 4A). Finally, from the sequence of four independent mutant alleles, we confirmed that *SDE1* was the *QDE-1* homolog (Figure 4B). Two of these alleles had single nucleotide deletions that disrupted an *SDE1* open reading frame (*sde1-1*: at position 1495 and *sde1-2*: at position 2955 of the transcribed sequence). A third allele had a 29 nt deletion (*sde1-3*) at position 1545 of the transcribed sequence. We also identified the fourth mutant allele (*sde1-4*) that could only be PCR amplified with primers from the 5′ end of *SDE1* (data not shown). Presumably, this allele had a deletion extending from the 3′ transcribed region into the adjacent flanking region of *SDE1*.

The sequence of *SDE1* cDNAs obtained by 3′ and 5′ RACE revealed that the SDE1 protein is 113.7 kDa (1196 aa) and is encoded in a 4182 nt mRNA. Comparison of the cDNA and genomic sequences revealed that there is one intron in the gene (424 nt at position 3127). From the partial genome sequence of *Arabidopsis*, we detected three additional SDE1 homologs. In addition, as in the analysis of QDE-1 and EGO-1 (Cogoni and Macino, 1999a; Smardon et al., 2000), we detected similarity with the RdRP of tomato (Schiebel et al., 1998) and with proteins encoded in *C. elegans* and *Schizosaccharomyces pombe* (Figure 4B). The plant sequences were all more similar to each other than to the animal and fungal homologs (S. R., unpublished data), indicating that the multiplicity of the plant homologs was generated after the evolutionary divergence of plants and other eukaryotes. The most conserved regions of the protein sequences are in the C-terminal regions between residues 420 and 1070 (Figure 4B). However, none of the sequence motifs in this region were recognized in other proteins of known function.

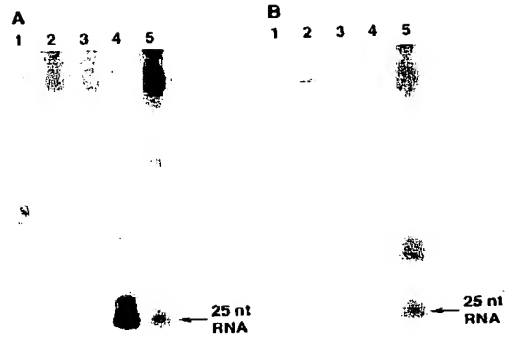

Figure 5. Northern Blot Analysis of 25 nt RNAs

Low molecular weight RNA was isolated from leaves of wild-type (wt) C24 (lane 1), wt A (lane 2), wt G (lane 3), wt[GxA] (lane 4), and *sde1*[GxA] (lane 5). Northern blot analysis was carried out using ^{32}P-labeled probes corresponding to the full-length *GFP* sequence (A) or the 3′ end of PVX (B).

25 Nucleotide RNA in an *sde1* Mutant

A predicted role of host-encoded RdRP in PTGS is in production of antisense RNAs that would anneal with and thereby mediate degradation of the target RNA (Kooter et al., 1999; Waterhouse et al., 1999). To test this idea, we assayed the wild-type and *sde1* mutant of GxA for the presence of 25 nt antisense RNAs corresponding to the 35S-*GFP* and 35S-*PVX:GFP* transgenes. We had previously shown that 25 nt RNAs corresponding to the sense and antisense polarity of transgene RNAs are invariably associated with PTGS (Hamilton and Baulcombe, 1999). These short RNAs are also produced in PVX-infected plants. They correspond to parts of the viral genome and are an indicator that protection against viruses is related to PTGS of transgenes. Based on the previous analysis of transgenic and virus-infected plants (Hamilton and Baulcombe, 1999), we predict that there would be three populations of 25 nt RNA in the wild-type GxA plants. One population would be derived from the replicating *PVX:GFP* RNA and the other two from the mRNA of 35S-*PVX:GFP* and 35S-*GFP* transgenes. The relative contribution of the 35S-*GFP* RNA-derived population would be indicated by the ratio of *GFP* and *PVX* sequences in the 25 nt RNA.

As expected, the 25 nt *GFP* or *PVX* RNAs were absent from nontransformed plants or line G in which PTGS was not active (Figure 5A, lanes 1 and 3; Figure 5B,

CAPS markers next to T9C5 and within T9C5 were used to locate *SDE1* within the 5′ 45 kb of T9C5. The number of recombination events among the number of chromosomes examined is shown at each marker.

(B) Alignment of the SDE1 protein with related sequences. The sequences displayed are tomato (tomato RdRp sequence EMBL: Y10403), athf10b6 (*Arabidopsis* RdRp homolog on BAC F10B6 EMBL: AC006917), athf2p3 (*Arabidopsis* EMBL: O82504), athf6f22 (*Arabidopsis* EMBL: O82190), atht9c5 (*Arabidopsis* SDE1), S. pombe (*S. pombe* EMBL: O14227), N. crassa (*N. crassa* QDE-1), and C. elegans (*C. elegans* EMBL: Q19285). Identity with the consensus is shown in black, similarity with the consensus is shaded in gray.

lanes 1 and 3). Also as expected, and consistent with a role in PTGS, the 25 nt *GFP* RNAs were abundant in wild-type GxA (Figure 5A, lane 4) (Dalmay et al., 2000). However, the 25 nt *PVX* RNAs were undetectable in that line (Figure 5B, lane 4). It is likely, therefore, that the 25 nt *GFP* RNAs in GxA were derived from the 35S-*GFP* mRNA rather than the replicating *PVX:GFP* RNA or the 35S-*PVX:GFP* mRNA. Consistent with that interpretation, the full-length viral *PVX:GFP* RNAs were not detectable in wild-type GxA (Figure 2).

In the *sde1* mutant, the *GFP* 25 nt RNAs were 6-fold less abundant than in the wild-type plants (Figure 5A, lane 5). In contrast, the *PVX* 25 nt RNAs, having been undetectable in the wild type, were more abundant (Figure 5B, lanes 4 and 5) in the mutant. We cannot formally rule out that these *PVX* 25 nt RNAs were derived from the 35S-*PVX:GFP* transgene mRNA. However, it is more likely that they were derived from the replicating *PVX:GFP* RNA that was present at elevated levels in the *sde1* mutant (Figure 2). If that is the case, the *sde1* mutation would be specific for PTGS induced by a transgene but not by a virus. The reduced level of the 25 nt *GFP* RNAs would be due to the absence of the populations derived from the mRNA of 35S-*PVX:GFP* and 35S-*GFP* transgenes and the low level of 25 nt *PVX* and *GFP* RNAs would be the population derived from the replicating *PVX:GFP* RNA.

sde Mutations Affect Transgene- but Not Virus-Induced Gene Silencing

To further investigate the possibility that SDE1 is required for transgene- but not virus-induced PTGS, we inoculated the crucifer strain of tobacco mosaic virus (crTMV) and tobacco rattle virus (TRV) to wild-type and *sde1* mutant lines of *Arabidopsis*. The pattern of initial TRV symptoms in *Arabidopsis* (data not shown) followed by "recovery" is diagnostic of PTGS (Ratcliff et al., 1997, 1999). In addition, from previous analyses, it was known that PTGS is a limiting factor in the accumulation of these viruses in infected plants (Voinnet et al., 1999).

If SDE1 is involved in virus-induced PTGS, we expected that the viral RNAs would be more abundant in the mutants than in the wild-type plants. Northern analysis (Figures 6A and 6B) showed however, that the *sde1* mutation did not affect accumulation of these viral RNAs: the viral genomic and subgenomic RNAs were equally abundant on the mutant and wild-type plants. Similar results were also obtained with plants infected with turnip crinkle virus (data not shown).

Our second test of virus-induced PTGS in the *sde1* mutant line exploited the ability of TRV vector constructs to silence expression of nuclear genes. For example, infection of wild-type *Arabidopsis* with a TRV vector harboring an insert of phytoene desaturase sequence caused a striking photobleached symptom. These symptoms were due to virus-induced PTGS of the endogenous phytoene desaturase gene leading to suppression of photoprotective carotenoid production (Figure 6C). The same phenotype developed on the *sde1* plants at the same rate as in the wild-type plants. Our interpretation of these symptoms, as with the 25 nt RNA (Figure 5) and viral RNA accumulation data (Figures 6A and 6B), is that the *SDE1* locus is not required for virus-induced PTGS.

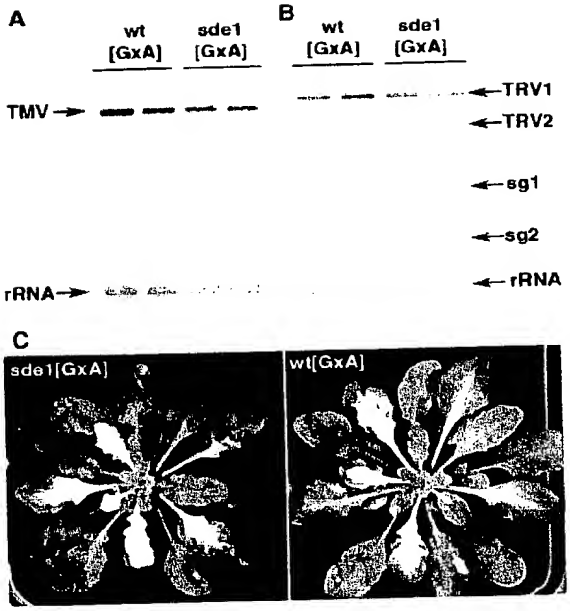

Figure 6. SDE1 Is Not Required for Virus-Induced Gene Silencing

RNA was isolated from leaves of crTMV- (A) or TRV- (B) infected wild-type (wt) [GxA] and *sde1*[GxA] plants. Northern blot analysis was carried out using ^{32}P-labeled probes corresponding to the 5′ part of crTMV (A top), to the 3′ part of TRV RNA1 (B top), and to rRNA (A and B bottom). The RNA species detected were the genomic RNA of crTMV (TMV), genomic (TRV1 and TRV2), or subgenomic (sg1 and sg2) length RNAs of TRV, and rRNA.

(C) Photographs were taken of TRV-PDS-infected *sde1*[GxA] and wt[GxA] plants, two weeks after inoculation. The white areas are a result of photobleaching due to the PTGS of the PDS gene.

Discussion

A Role for RdRP in PTGS

We have used mutagenesis of *Arabidopsis* to identify four genetic loci controlling PTGS. One of these, *SDE1*, has been cloned and, based on mutant phenotype and sequence, we conclude that the SDE1 protein carries out the same PTGS role as the QDE-1 protein of *N. crassa* (Cogoni and Macino, 1999a). This role is likely to involve RdRP activity since QDE-1 and SDE1 share extensive similarity with the RdRP of tomato (Schiebel et al., 1998).

The role of an RdRP in PTGS was predicted several years ago (Lindbo et al., 1993) and more recently various PTGS models have been proposed in which this enzyme uses a double-stranded RNA template (Kooter et al., 1999; Waterhouse et al., 1999). Consistent with these models, it is well established that dsRNA is the initiator of RNA interference in animals (Fire et al., 1998; Montgomery et al., 1998; Sharp, 1999). In plants, a role of dsRNA could explain why inverted repeat transgenes and coexpressed sense and antisense RNA can induce PTGS (Stam et al., 1997; Hamilton et al., 1998; Waterhouse et al., 1998): the readthrough transcripts of the inverted repeats could form intramolecular RNA duplexes and the sense and antisense RNAs could form

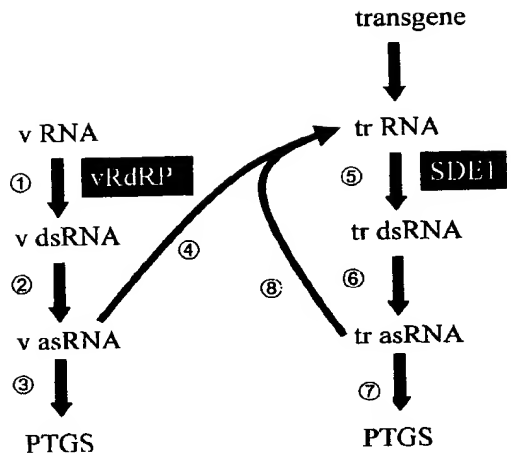

Figure 7. A Schematic Representation of PTGS in GxA

The GxA line harbors three mediators of PTGS. There are two transgenes (35S-*GFP* and 35S-*PVX:GFP*) and the replicating *PVX:GFP* (vRNA). The v RNA is copied into double-stranded viral RNA (v dsRNA) by the virus-encoded RNA-dependent RNA polymerase (v RdRP). The v dsRNA in turn is processed to antisense RNA (v asRNA) that mediates PTGS. In GxA and the parental line A, the PTGS is weak because the PVX replication is inefficient in *Arabidopsis*. The v as RNA is also able to anneal to the sense RNA transcript of the transgenes (tr RNA) and prime SDE1-dependent synthesis of double-stranded RNA tr dsRNA. The tr dsRNA is then processed into antisense RNA (tr asRNA) that mediates PTGS and is also able to anneal with the tr RNA. The numbers 1–8 refer to different stages in the process as described in the main text.

intermolecular duplexes. Similarly, the dsRNA intermediate in virus replication could explain why RNA viruses are efficient initiators of PTGS (Ratcliff et al., 1997, 1999; Ruiz et al., 1998).

However, the models in which dsRNA is an RdRP template are not consistent with the analysis described here in which SDE1 is required for PTGS induced by a transgene, but not by a replicating *PVX:GFP* RNA (Figures 5 and 6). If dsRNA is the template of the SDE1 RdRP, then *sde1* mutations should affect PTGS in both transgenic and virus-infected plants. Our alternative explanation is that the SDE1 RdRP does not use the dsRNA as template but that it is responsible for its synthesis. In this scenario, virus- and transgene-induced PTGS would use different mechanisms for production of double-stranded RNA. In transgenic plants, the production of dsRNA would be SDE1 dependent whereas in virus-induced PTGS, it would be synthesized by a viral RdRP and independent of SDE1.

An SDE1-Mediated Cycle of PTGS

In GxA, the PTGS is due to a combination of replicating *PVX:GFP* RNA and transgene RNA (Dalmay et al., 2000). The PTGS from the 35S-*PVX:GFP* and 35S-*GFP* transgene RNAs would be dependent on SDE1. In contrast, the replicating *PVX:GFP* viral RNA (encoded by the 35S-*PVX:GFP* transgene) would mediate PTGS in an SDE1-independent manner. Figure 7 provides a schematic representation of interactions between the viral RNA and the transgenes that would lead to PTGS. The first stage in this scheme would be viral RdRP-dependent synthesis of ds*PVX:GFP* RNA as a virus replication intermediate (Figure 7, stage 1). The presence of this dsRNA would then lead to the production of short antisense RNAs (Figure 7, stage 2). A precedent for this process could be the processing of dsRNA in extracts of *Drosophila* cell cultures that exhibit aspects of RNAi in vitro (Zamore et al., 2000). The short RNAs could provide specificity to a process of RNA degradation, also as in an in vitro system of RNAi (Hammond et al., 2000), and thereby account for PTGS by the 35S-*PVX:GFP* transgene (Figure 7, stage 3). In addition, the antisense RNAs could anneal with sense *GFP* RNA from the 35S-*GFP* transgene (Figure 7, stage 4) and, assuming that SDE1 requires a primer, mediate the SDE1-dependent synthesis of double-stranded RNA (Figure 7, stage 5).

In wild-type plants the SDE1-dependent process would operate as a cycle because the antisense RNA produced from the *GFP* dsRNA (Figure 7, stage 6) would be functionally equivalent to the *PVX:GFP* antisense RNA (Figure 7, stage 4): it would mediate PTGS (Figure 7, stage 7) and it could prime secondary production of dsRNA by annealing to the sense *GFP* RNA from the 35S-*GFP* transgene (Figure 7, stage 8). A key feature of this scheme is that, as in many examples of PTGS, there are separate genetic elements responsible for initiation and maintenance. The viral RNA would mediate initiation (Figure 7, stages 1–3) and the transgenes would mediate the maintenance stages (Figure 7, stages 5–8) of the PTGS process. A second important feature of this model is the ability of the maintenance process to be self-sustaining through the secondary production of dsRNA by annealing to the sense *GFP* RNA from the 35S-*GFP* transgene (Figure 7, stage 8).

The biochemical properties of plant-encoded RdRP are largely consistent with the scheme proposed in Figure 7. The sequence of the protein is similar to that of SDE1 (Schiebel et al., 1998) and the in vitro product is double-stranded (Schiebel et al., 1993a; Schiebel et al., 1993b). There is no evidence that the plant RdRP requires a primer in vitro as indicated in Figure 7. However, the sequence of the biochemically characterized enzyme (Schiebel et al., 1998) is more closely related to other *Arabidopsis* homologs than to SDE1 (S. R., unpublished data) and it remains possible that there is functional variation in the different forms of RdRP. It is also possible that the RdRP requires a primer in vivo but not in vitro.

Separate Initiation and Maintenance Stages of PTGS

This differentiation of initiation and self-sustaining maintenance stages, as indicated in Figure 7, is a feature of many examples of PTGS. For example, in virus-induced PTGS the presence of the virus is not required once the process has been initiated (Ruiz et al., 1998). Similarly, when a graft-transmissible signal initiates PTGS of a highly expressed transgene, the PTGS phenotype persists even after the graft union is broken (Palauqui and Vaucheret, 1998; Voinnet et al., 1998). In both of these examples, as in *Arabidopsis* line GxA, the transgene would maintain the process once it had been initiated. The initiators would be the viral RNA or the silencing signal molecule. The SDE1 cycle proposed in Figure 7

could also explain aspects of RNA interference in *C. elegans* (Fire et al., 1998). There is clearly an amplification step in RNA interference that could be similar to the maintenance of PTGS in plants (Fire et al., 1998). In addition, as in plants, an RdRP homolog (EGO-1) is required for RNA interference in *C. elegans* (Smardon et al., 2000).

Although a self-sustaining cycle involving an RdRP may be important in many examples of PTGS, there may also be examples in which this process does not operate. For example, as with virus-induced PTGS described here (Figures 5 and 6), if the production of dsRNA does not require a host-encoded RdRP there would be no requirement for *SDE1*. Thus, when dsRNA is produced as a direct result of readthough transcription of inverted repeat transgenes (Stam et al., 1997, 1998), the PTGS may be SDE1 independent. Similarly, with antisense transgenes, the PTGS could be maintained in an SDE1-independent manner if dsRNA is generated by annealing of an antisense transgene transcript with the sense RNA of another transgene (Waterhouse et al., 1998).

Strong and Weak PTGS with Different Transgenes

The proposed involvement of the 35S-*GFP* and 35S-*PVX:GFP* transgenes and the replicating *PVX:GFP* RNA accounts for different levels of PTGS in wild-type GxA, *sde1* mutant GxA, and in the wild-type line A. Thus, in wild-type GxA, the PTGS would be strong because it would be initiated by the replicating *PVX:GFP* RNA and maintained by the 35S-*GFP* transgene. This transgene is transcribed at a high level and would provide an abundant substrate for SDE1 (Figure 7). In the parental line A, the PTGS is weaker than in GxA because the two sources of PTGS (the 35S-*PVX:GFP* transgene RNA and the replicating *PVX:GFP* RNA) are both weak silencers. With the 35S-*PVX:GFP* transgene, the PTGS is weak because most of its transcripts terminate prematurely (Dalmay et al., 2000). The replicating *PVX:GFP* RNA is a weak silencer because it replicates only inefficiently in *Arabidopsis*. However, the weakest silencing of all of the examples described here is in the *sde1* mutant GxA. The mutant phenotype would mean that there would be no PTGS due to the 35S-*GFP* and 35S-*PVX:GFP* transgenes. Thus, the only effective silencing agent in these plants would be the inefficiently replicating *PVX:GFP* RNA.

The relationship between these different elements in the process is complicated by the potential of the various RNA species to be both mediators and targets of PTGS. Nevertheless, there is the expected relationship between the level of PTGS and the level of viral *PVX:GFP* RNA. Thus, in wild type GxA when the PTGS is strongest, the viral *PVX:GFP* RNA is almost undetectable (Figure 2) whereas in the *sde1*mutant, when the PTGS is weakest, the viral *PVX:GFP* RNA is at its highest level (Figure 2). Line A represents an intermediate level of PTGS and a correspondingly intermediate level of viral *PVX:GFP* RNA (Figure 2).

The Effect of SDE1 on Transgene Methylation

In our previous characterization of GxA, we noted that transgene methylation depends on the combined presence of the 35S-*PVX:GFP* and the 35S-*GFP* transgenes (Dalmay et al., 2000). We proposed that the presence of replicating *PVX:GFP* RNA initiates PTGS and leads, either directly or indirectly, to transgene methylation through an RNA–DNA interaction. Now, with the finding that the transgenes in the *sde* mutants are not methylated despite the presence of viral *PVX:GFP* RNA (Figures 2 and 3), we conclude that the transgene methylation is not directly due to viral *PVX:GFP* RNAs. A more likely possibility is that transgene methylation is mediated by the 25 nt *GFP* RNAs. In this scenario, the viral *PVX:GFP* RNA would participate indirectly in the proposed RNA–DNA interaction via the SDE1 cycle as depicted in Figure 7.

The involvement of the 25 nt RNAs is supported by the finding that the 35S-*GFP* and 35S-*PVX:GFP* transgenes were methylated only in the wild-type GxA line, when the 25 nt *GFP* RNAs were most abundant (Figure 5). In all other samples, including the parental lines G and A, or in the *sde1* GxA mutant, the transgenes were not methylated and the 25 nt *GFP* RNAs were less abundant than in the wild-type GxA. There is also coincidence of sequence specificity: the 25 nt RNA species in GxA are specific to *GFP* rather than *PVX* sequences (Figure 5) and, likewise, the 35S-*PVX:GFP* transgene is methylated in the *GFP*- rather than the *PVX*-specific regions (Dalmay et al., 2000).

However, we still do not know the role of transgene methylation in PTGS. We had suggested previously that examples of PTGS with an epigenetic component could be identified by transgene methylation (Jones et al., 1999; Dalmay et al., 2000). This possibility cannot be ruled out. However, as there could be an SDE1-dependent cycle of PTGS operating at the RNA level (Figure 7), we can now appreciate that an epigenetic effect need not involve changes at the DNA or chromatin level. If the methylation does have a causal role in the gene silencing mechanism, it could be in a secondary process that reinforces the proposed SDE1-dependent PTGS cycle.

Natural Roles of PTGS

The role for PTGS in defense against viruses and transposons is well established (Ratcliff et al., 1997, 1999; Ketting et al., 1999). Other roles, for example in development and basic cellular function, have been proposed (Voinnet et al., 1998). SDE1 is not required for virus-induced PTGS and it is unlikely to be involved in the antiviral defense role of PTGS. Similarly, a role in development or basic cellular function is also unlikely because the *sde1* plants grow and develop normally. A more likely possibility is that *SDE1* is required for protection against transposable DNA, as for the *MUT7* PTGS locus in *C. elegans* (Ketting et al., 1999).

None of the other three *SDE* loci are likely to have a role in development or basic cellular function because, like the *sde1* mutants, the plants grow and develop normally. The *sde2* and *sde3* mutants have a PTGS phenotype that is similar to *sde1*, indicating they are also involved in the stage of the PTGS mechanism providing protection against transposons. These genes could encode proteins that are involved, for example, in stabilizing sense/antisense RNA complexes (Figure 7, stage 8) or, perhaps, they are subunits of an RdRP complex. It

has been noted previously that plant-encoded RdRPs including SDE1 do not have the characteristic motifs of RNA polymerases and that the holoenzyme may include additional, as yet uncharacterized proteins (Baulcombe, 1999).

It is striking that out of 11 mutant alleles characterized in this study, there is only one (*sde4*) with a distinct phenotype indicating that it may not act at the same stage in the PTGS mechanism as SDE1. We have yet to characterize this mutant in detail. However, our failure to identify large numbers of mutants with, for example, defective RNA targeting or signaling of PTGS may indicate that there are parts of the PTGS mechanism with roles in development or basic cellular function. If that is the situation, the fast neutron mutagenesis used here may be inappropriate for the study of these functions because it generates deletion mutants with a null phenotype. To complete the mutagenesis of PTGS, it may be necessary to produce point mutations and develop conditional mutant screens.

Experimental Procedures

Transgenic Plants and Mutagenesis

The wt A, wt G, and wt[GxA] *Arabidopsis* (C24) were described (Dalmay et al., 2000) as Amp243, GFP142, and GFP142xAmp243, respectively. The wt A contains a *35S-PVX:GFP* and wt G carries a *35S-GFP* transgene. Both lines are homozygous and have a single copy of the respective transgene. The wt[GxA] is the progeny of a cross between the two lines above and it is homozygous for both of the transgenes. 10,000 seeds from [GxA] were mutagenized by exposure to fast neutrons at the International Atomic Energy Agency (Vienna, Austria) using a 60 Gy unit. The M1 seedlings were pooled into 50 pools of 200 plants. 3000 M2 plants were screened from each pool.

GFP Imaging

GFP expression was monitored using an MZ12 dissecting microscope (Leica, Heidelberg, Germany) coupled to an epifluorescence module. Photographs were taken using Kodak Ektachrome Panther (400 ASA) film.

RNA and Nuclear Run-Off Transcription Analysis

RNA gel blot analysis was performed as described previously (Mueller et al., 1995). DNA fragments were labeled by random priming incorporation of ^{32}P-dCTP (Amersham). After hybridization, the signal present in the membranes was analyzed and quantified using the Fujix Bio-Imaging Analyzer Bas 1000 (Fuji Photo Film Co., Ltd., Fuji, Japan) equipment. Polymerase chain reaction, amplified, full-length *GFP* DNA was used for the GFP specific probe. The crTMV specific probe was generated using a 3 kb DNA fragment from the 5′ end of crTMV. TRV RNA was detected with a probe made of a BstEII and SmaI fragment (5345–6792) of a TRV1 clone (pTR7116) (Hamilton and Baulcombe, 1989) which can hybridize weakly with TRV RNA2 due to the approximately 300 nt sequence identity at the 3′ end of RNA1 and RNA2.

The 25 nt RNAs were extracted and probed as described (Hamilton and Baulcombe, 1999; Dalmay et al., 2000). Full-length *GFP* cDNA or a 1 kb fragment from the 3′ end of PVX RNA (containing the coat protein and 3′ UTR) was used to generate the probes.

Nuclei for run-off transcription analysis were isolated as described (Covey et al., 1997), and incorporation of uridine 5′-^{32}P-triphosphate (Amersham) was determined by probing 1 μg of the appropriate DNA samples immobilized as slots on Hybond-N+ membranes (Amersham). Incorporation was assessed by using a Fujix Bio-Imaging Analyzer BAS 1000 (Fuji Photo Film Co., Ltd., Fuji, Japan) as described (Mueller et al., 1995).

DNA Analysis

Genomic DNA was extracted from leaves, and Southern blot analysis was performed as described (Jones et al., 1999). The ^{32}P-labeled DNA probes corresponded to the entire 812 nt of *GFP* cDNA.

Genetic Mapping and DNA Sequence Analysis

A set of CAPS markers described by Konieczny and Ausubel (1993) was used to detect polymorphism between the Columbia and Landsberg ecotypes and to map *sde* loci to the 10 chromosome arms. However, several of these markers did not show polymorphism between C24 and Landsberg ecotypes and in some instances it was necessary to generate alternative markers. The detail of those markers is available on our website (http://www.jic.bbsrc.ac.uk/science/index.htm).

The *sde1* mutation was located adjacent to *GL1* (nine recombination events) and *F3H* (two recombination events) in a screen of thirty mutants and thirty wild-type plants in an F2 mapping population. To locate the map position of this locus more precisely, we generated three markers at the positions of *ASN1*, r30025, and *AP3La* and the F2 mapping population was expanded to 150 plants. The screen with these three markers and *F3H* allowed us to conclude that *SDE1* is approximately halfway between *ASN1* and r30025 in a region that had been cloned and sequenced as part of the Arabidopsis Genome Sequencing Project (http://www.genoscope.cns.fr).

Using the BLAST program (Altschul et al., 1990) we identified a *QDE-1* homologous sequence in the BAC clone, T9C5 (accession number EMBL: AL132964), in the region close to *SDE1*. Using an F2 mapping population of 610 plants and another 8 CAPS markers, the *SDE1* locus was positioned between 1 and 45,000 bp on the T9C5 BAC clone. The sequence of the CAPS marker primers is available on request.

Five overlapping DNA fragments were generated by PCR from the wt, *sde1-1*, *sde1-2*, and *sde1-3* plants at the position in BAC T9C5 positions 22,000–30,000 kb and sequenced directly using the Big Dye Terminator Mix (PE Applied Biosystem). The sequencing reactions were resolved on an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, USA). The regions where mutation was found were sequenced on both strands from three independent PCR reactions from both the wt and the mutant plants.

The 5′ and 3′ ends of the *SDE1* cDNA were determined by rapid amplification of cDNA ends (RACE) by using the Marathon cDNA amplification kit (Clontech). RACE products were cloned into pGEM-T plasmid (Promega) and sequences of 10 independent 3′ and 5′ end clones were determined. The cDNA library, obtained by the Marathon cDNA amplification kit (Clontech), was used as a template to PCR amplify five overlapping fragments that were directly sequenced. The number and location of intron(s) was determined by comparing the sequence data obtained from the genomic DNA with the data obtained from the cDNA.

Other protein sequences homologous to SDE1 were identified using the BLASTP program (Altschul et al., 1990). Protein sequences were obtained and collated using the Wisconsin package (Wisconsin Package Version 10.0, Genetics Computer Group [GCG], Madison, WI). Sequence alignments were produced using CLUSTALW (Thompson et al., 1994) and were displayed using PRETTYBOX from the Wisconsin package.

The sequence data of the *SDE1* cDNA has been deposited in the GenBank database (Accession number AF268093). The sequence of the mutants is available on our website (http://www.jic.bbsrc.ac.uk/welcome.htm).

Wild-Type and Recombinant Viruses

The crTMV strain that infects *Arabidopsis* was obtained from Dr. J. Atabekov (Dorokhov et al., 1994) and the PPK20 strain of TRV from Dr. J. Bol (Leiden University). The TRV vector carrying the phytoene desaturase fragment is similar to TRV:GFP described previously (Ratcliff et al., 1999) and will be described in more detail elsewhere (F. Ratcliff, A. Montserrat Martin Hernandez, and D. C. B., unpublished data). The insert in RNA2 of the TRV:phytoene desaturase vector is 1770 nt of the *Arabidopsis* phytoene desaturase sequence.

Acknowledgments

We thank John Bol and Joseph Atabekov for viral strains. Frank Ratcliff and Ana Montserrat Martin Hernandez provided access to

unpublished tobacco rattle virus vector clones for which we are especially grateful. We are also grateful to Andy Maule for discussions and guidance. S. R. received a Ph.D. studentship from the John Innes Foundation. Financial support was from the Gatsby Charitable Foundation, the European Community framework IV COMREP project, and the BBSRC (grant 83/GAT09114). The work with modified and imported viruses was carried out under licence from the Ministry of Agriculture, Fisheries, and Food (PHL 24A/2921).

Received February 24, 2000; revised April 11, 2000.

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GenBank Accession Number

The sequence data of the *SDE1* cDNA reported in this paper has been deposited in the GenBank database with accession number AF268093.

Encl. 2

RESEARCH COMMUNICATION

Involvement of small RNAs and role of the *qde* genes in the gene silencing pathway in *Neurospora*

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Small RNA molecules have been found to be specifically associated with posttranscriptional gene silencing (PTGS) in both plants and animals. Here, we find that small sense and antisense RNAs are also involved in PTGS in *Neurospora crassa*. The accumulation of these RNA molecules depends on the presence of functional *qde-1* and *qde-3* genes previously shown to be essential for gene silencing, but does not depend on a functional *qde-2*, indicating that this gene is involved in a downstream step of the gene silencing pathway. Supporting this idea, a purified QDE2 protein complex was found to contain small RNA molecules, suggesting that QDE2 could be part of a small RNA-directed ribonuclease complex involved in sequence-specific mRNA degradation.

Received December 10, 2001; revised version accepted February 11, 2002.

Posttranscriptional gene silencing (PTGS) occurs in fungi, plants, and animals as a response to various types of foreign nucleic acids including transgenes, transposons, viral RNAs, and double-stranded RNA (dsRNA; Baulcombe 1999; Bosher and Labouesse 2000; Cogoni and Macino 2000; Waterhouse et al. 2001; Zamore 2001). In this process RNA molecules with sequence similarity to the introduced nucleic acid are degraded in a sequence-specific manner. Gene silencing is thought to represent an ancient natural defense system against viruses and transposons that has been conserved through evolution (Matzke et al. 2000). Recent studies have, indeed, indicated that the molecular basis of the PTGS mechanism is similar in different organisms. In particular, genetic screens carried out in *Neurospora crassa*, *Arabidopsis thaliana*, and *Caenorhabditis elegans* have identified homologous genes required for PTGS (Cogoni and Macino 1997; Ketting and Plasterk 2000; Mourrain et al. 2000). An emerging common model for PTGS is that transgenes or transposons are the source RNA molecules that are recognized by the silencing machinery and that subsequently activate a cascade of events leading to sequence-specific mRNA degradation. A key step in this process is likely to involve RNA-dependent RNA polymerases (RdRPs) that have been found to be required for PTGS in plants (Dalmay et al. 2000; Mourrain et al. 2000), fungi (Cogoni and Macino 1999a), and animals (Smardon et al. 2000). It has been proposed that the RdRP can recognize RNAs produced from transgenes or transposons and convert them into dsRNAs. Recent findings, obtained using cell-free extracts of *Drosophila*, indicate that dsRNA is processed into small RNA molecules of 21–23 nucleotides by an enzyme called Dicer, which contains both an RNase III and an RNA helicase domain (Zamore at al. 2000; Bernstein et al. 2001). Similar-sized small RNAs have also been associated with transgene-induced silencing in plants (Hamilton and Baulcombe 1999), suggesting that these RNAs play a central role in PTGS. Moreover, the introduction of these small RNAs (also called siRNA, short interfering RNA) in mammalian cells has been observed to specifically suppress the expression of endogenous genes, suggesting that siRNAs can induce sequence-specific RNA degradation (Elbashir et al. 2001). The hypothesis that siRNAs can work as guide molecules in inducing RNA degradation is further supported by the finding that siRNAs are associated with the *Drosophila* RNA-induced silencing complex (RISC), showing a sequence-specific RNAse activity that degrades mRNAs homologous to the silencing trigger (Hammond et al. 2000). Recently, one of the proteins belonging to the RISC multicomponent nuclease has been characterized and found to be a member of the Argonaute family of proteins (Hammond et al. 2001), which was previously found to be essential for PTGS in *Neurospora* (Catalanotto et al. 2000), *C. elegans* (Tabara et al. 1999), and *Arabidopsis* (Fagard et al. 2000). This observation suggests that similar siRNA-directed nuclease complexes might be conserved in eukaryotic organisms belonging to all three kingdoms. Moreover, it also indicates that different foreign nucleic acid molecules, either transgenic DNA or dsRNA, can converge to activate a similar RNA-degrading machinery.

In this work, to establish the universality of the PTGS mechanism, we looked for siRNAs in *Neurospora crassa*. We detected short sense and antisense RNAs 25-nt long that specifically accumulated in silenced transgenic strains of *Neurospora*. Moreover, we found that siRNAs derive from the vector portion of the transgenic construct, indicating that the transgenic transcripts are recognized by the silencing machinery, converted into dsRNA, and then diced into small RNA molecules. Additionally, we show that a functional *qde-2* (quelling defective) gene (Catalanotto et al. 2000), although essential for PTGS, is not necessary for siRNA accumulation, suggesting that QDE2 could be required in an siRNA-directed RNA-degradation step. Supporting this role of QDE2 in the mRNA-degradation step, we found that the QDE2 protein copurifies with siRNAs, which suggests that QDE2 is a component of the siRNA-directed nuclease complex.

Results and Discussion

To investigate the PTGS mechanism in fungi, we looked for the presence of small RNAs in an *N. crassa* strain in

[*Key Words*: Posttranscriptional gene silencing; double-stranded RNA; short interfering RNA; quelling defective; small temporal RNA; RNA-induced silencing complex]

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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.222402.

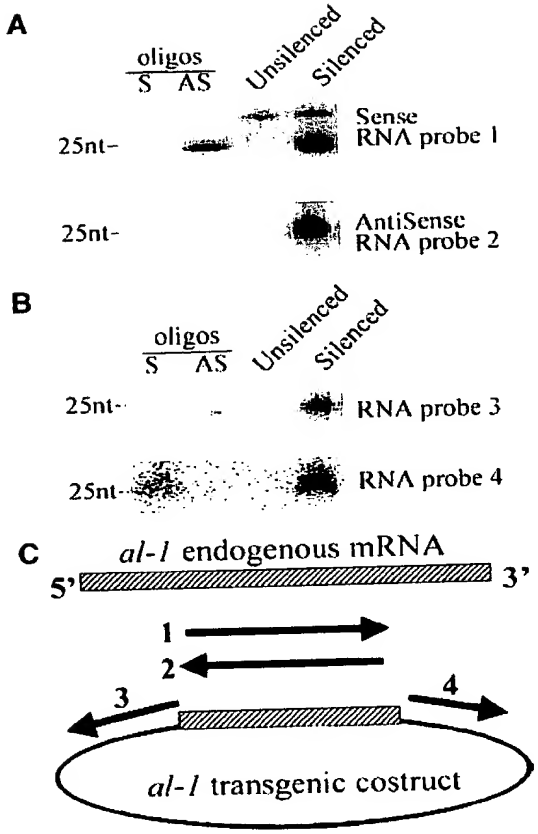

Figure 1. Small 25-nt sense and antisense RNAs derived fr transgenic transcripts are associated with PTGS. RNA blot hyb ization: 50 µg (each lane) of enriched low-molecular-weight RI prepared from either untransformed wild-type (unsilenced) or ... - silenced strain (6xw). Identical RNA blots were hybridized with the following RNA probes: (*A*) Probes 1 and 2 corresponding to the duplicated *al-1* region that are able to recognize small RNAs derived from both the transgenic and endogenous *al-1* gene. The presence of the upper bands in wild-type and 6xw lanes were due to nonspecific hybridization of the RNA probe. (*B*) Probes 3 and 4 were, instead, specific for the transgenic chimeric RNA. 30 pmoles per lane of 25-nt DNA oligonucleotides in sense (S) and antisense (AS) orientation were used to verify the hybridization specificity and also as size markers in both *A* and *B*. (*C*) Schematic representation of plasmid pal-1.6 and RNA probes.

which the endogenous gene *albino 1* (*al-1*) is posttranscriptionally silenced as a consequence of the introduction of cognate transgenes. By using both sense and antisense RNA probes corresponding to the region of the *al-1* gene present in the transgenic construct (Fig. 1A,C), we found that small RNA molecules in both sense and antisense polarity with a size of ~25 nt were present in the *al-1* silenced strain but not in a wild-type unsilenced strain. This result provides evidence that small RNA intermediates are a general feature of posttranscriptional gene silencing. Although the introduced transgenic construct does not contain a promoter, previous results (Cogoni et al. 1996) indicated that unexpected transcription of transgenic loci was associated with the occurrence of gene silencing. In particular, chimeric transgenic transcripts containing sequences corresponding both to the *al-1* gene and to the bacterial vector were specifically found in silenced strains (Cogoni et al. 1996). These chimeric transcripts could be recognized by the cell as aberrant and therefore able to induce the cascade of events leading to RNA degradation. To analyze if chimeric transgenic transcripts are, indeed, templates for production of sense/antisense small RNA, we used vector sequences as probes (Fig. 1C). We found that small RNAs corresponding to the vector sequences are specifically accumulated in the silenced strains (Fig. 1B). Moreover, the small RNAs were found in both sense and antisense polarity, suggesting that, to produce siRNAs, transgenic sense RNA transcripts should first be converted to dsRNA. Then, in keeping with findings in other systems (Hammond et al. 2001), the dsRNA is likely to be pro- ... into small sense/antisense RNA by an RNase III ... *Drosophila*. The fact ... detect ...ecting ...t indi- ...roduc- ... by mu- ...encode ... Cogoni ...*le* genes ...ence of ...t muta- ...*e-2* gene ...ggesting ...involved ... in steps ...is finding ...wo genes. ...polymer- ...has been ...n of anti- ...s, leading ...sequently ... to the ... ...ce activity processed into small RNAs by a RNase ... (Bernstein et al. 2001). The role of *qde-3*, which encodes a DNA helicase (Cogoni and Macino 1999b), has been proposed to be involved in a nuclear step necessary for the activation of gene silencing, perhaps by modifying

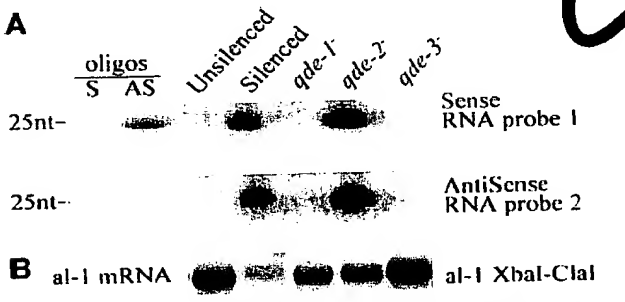

Figure 2. Analysis of small RNA accumulation in *qde* mutants. (*A*) RNA blot hybridization of low-molecular-weight RNAs prepared from the 107 strain (*qde-1*$^-$), the 820 strain (*qde-2*$^-$), the 627 strain (*qde-3*$^-$), the untransformed wild-type strain (unsilenced), and an *al-1* silenced strain (6xw). Sense and antisense probes are the same as in Figure 1. (*B*) Northern blot on total RNA extracts hybridized with a probe specific for *al-1* mRNA.

the chromatin state of the transgenic loci, allowing for the production of the silencing RNA signal.

The fact that in a *qde-2* mutant background the *al-1* mRNA is not degraded even in the presence of the same amount of small RNA found in a silenced strain (Fig. 2) indicates that the *qde-2* gene product is not necessary for siRNA production but is essential for some steps required in the sequence-specific RNA-degradation process guided by siRNAs. Consistent with this hypothesis, AGO2, a homolog of QDE2, has recently been found to be a component of a multiprotein complex (RISC; Hammond et al. 2001) that is required in dsRNA-induced gene silencing (also called RNA interference, RNAi) in *Drosophila*. Purified RISC complexes were found to retain the ability to induce degradation of target RNAs and to contain small RNAs that are hypothesized to confer sequence specificity to the ribonuclease complex (Hammond et al. 2000).

We tested whether QDE2, like its *Drosophila* homolog AGO2, is associated with small RNAs in the posttranscriptional mechanism in *Neurospora*. With this aim, we purified the QDE2 protein complex from a *Neurospora* strain expressing a Flag-epitope-tagged version of QDE2. We transformed an *al-1* transgenic strain (820) in which the silencing of the *al-1* gene was released by a deletion of the *qde-2* gene, with a plasmid containing the *qde-2* gene tagged at its N terminus with the Flag epitope and under the control of its own promoter. Several strains showing the recovery of *al-1* silencing were isolated, indicating that Flag–QDE2 functions in gene silencing as the wild-type QDE2 does in vivo. The expression of the Flag–QDE2 was confirmed by Western analysis using an anti-Flag antibody on extracts from two independent transgenic lines (Fig. 3A). Moreover, the Flag–QDE2 was also recognized by antibodies against QDE2, and appears to be roughly the same size as the wild-type QDE2 (Fig. 3A). We purified Flag–QDE2 from *Neurospora* whole-cell extracts by immunopurification with anti-Flag affinity gel beads and elution by competition with Flag peptide. Protein extracts were prepared from an *al-1* silenced strain expressing Flag–QDE2 and in parallel, as negative control, from two *al-1* silenced strains (6xw) containing a native un-Flagged version of QDE2. SDS-PAGE and silver staining revealed several proteins specifically present in the immunopurification (IP) from the strains expressing Flag–QDE2 (data not shown). Furthermore, three of these bands were recognized by immunoblotting analysis using an anti-QDE2 antibody (Fig. 3B). Presumably, the species with the lowest mobility corresponds to the full-length Flag–QDE2, and the two faster-migrating species could be produced by proteolysis of the full-length form. The same three species were also detected by using a QDE2-specific antibody. In contrast, the native QDE2 protein of the 6xw strain was only detected in flowthrough of the IP but not in IP eluates, indicating that native QDE2 protein was not bound by Flag gel beads.

To verify whether small RNA molecules were associated with the QDE2 protein, immunopurified protein fractions from both the Flag–QDE2-expressing strain and the silenced (6xw) strain were used to extract RNA, which was then analyzed by Northern blotting with an *al-1* riboprobe. We found that 25-nt RNAs corresponding to the *al-1* gene were present in the eluate of the Flag–QDE2-expressing strain; in contrast, these RNAs were not detected in the eluate of the silenced strain (Fig. 3C).

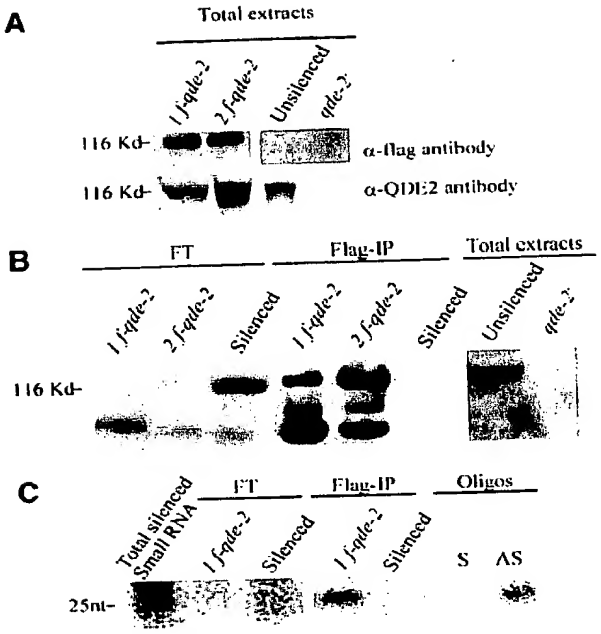

Figure 3. QDE2 copurifies with small RNAs. (*A*) Characterization of antibodies. α-Flag monoclonal and α-QDE2 antibodies were used in Western analysis of total proteins extracted from two deletion *qde-2* mutants that express Flag–QDE2 protein (lines 1f-qde-2 and 2f-qde-2), a wild-type (unsilenced) strain, and a *qde-2* deletion mutant strain 820 (*qde-2*⁻). The estimated protein size is shown on the left. (*B*) QDE2 protein was immunopurified with anti-FLAG affinity gel beads from protein extracts of two Flag–QDE2 strains and of the *al-1* silenced strain 6xw (silenced). Western analysis, using as probe α-QDE2 antibody, was performed on the flowthrough fraction (FT) and on the immunopurified fraction (Flag-IP). Three bands were specifically detected only in immunopurified fractions of Flag–QDE2-expressing strains (lines 1f-qde-2 and 2f-qde-2). The slower-migrating band comigrates with the wild-type version of QDE2 (unsilenced), total extract, indicating that the two additional faster-migrating bands could be proteolyzed forms produced in the IP elution step. A band corresponding to QDE2 is present in the flowthrough (FT) fraction of the silenced (6xw) strain only, indicating that the wild-type QDE2 is not bound by the anti-Flag affinity gel beads. (*C*) Small RNA molecules were extracted from the Flag-IP protein fractions and analyzed by Northern blotting using a riboprobe specific for the *al-1* sequence. Small RNAs of ~25 nt were detected in the RNA preparations from the immunopurified fraction of the Flag–QDE2 strain (line Flag-IP/1f-qde-2) but not in the 6xw strain (Flag-IP/6xw). In contrast, small RNAs are detected in the flowthrough of 6xw (FT/Silenced) but not in the Flag–QDE2 strain (FT/1f-qde-2). The weakness of the signal in the flowthrough fraction recovered from the 6xw strain is explained by the high dilution of this fraction. A small-RNA-enriched preparation from the total RNA extracted from the 6xw strain is used as a positive control. Sense (S) and antisense (AS) 25-nt oligonucleotides are used as size markers.

Moreover, small RNAs were detected in the flowthrough fraction of the 6xw strain, indicating that siRNAs copurify with QDE2.

On the whole, our results are in accordance with other findings obtained in *Drosophila* in vitro systems, which indicate that AGO2, a homolog of QDE2, is one constituent of a multicomponent nuclease complex involved in RNAi and also contains small RNA as cofactors (Hammond et al. 2001). Furthermore, members of

this protein family occur in almost all the eukaryotes, and several of them are implicated in gene silencing. In addition to *qde-2* of *Neurospora* and *ago-2* of *Drosophila*, *rde-1* is required for RNAi in *C. elegans* (Tabara et al. 1999), and *argonaute 1* (*ago-1*) is involved in PTGS in Arabidopsis (Fagard et al. 2000). Although the role of the QDE2/AGO2/RDE1/AGO1 family in gene silencing is well established, their biochemical function is still unknown, as is also still unclear whether they participate in the same step of the silencing pathway. These proteins have in common two motifs: a domain called PAZ and a domain called PIWI. The function of both these domains has yet to be defined; however, it has been suggested that the PAZ domain (Cerutti et al. 2000), which is also contained in Dicer, could be involved in mediating protein–protein interactions occurring between Dicer and AGO2 (Hammond et al. 2001). Using an approach combining genetic and biochemical analysis, we found that QDE2 is dispensable for siRNA accumulation but required for steps in posttranscriptional gene silencing in which siRNA are used as cofactors to direct sequence-specific degradation. However, further studies will be required to understand if small RNAs interact directly with QDE2 or if they are bound to another protein belonging to the QDE2 protein complex and that copurifies with QDE2. Of particular interest is that the components and biochemical mechanism underlining the targeting and degradation of mRNA appear to be very similar in gene silencing phenomena induced either by trangenes as in *Neurospora* or by dsRNA as in *Drosophila*. Therefore, the emerging view is that different gene silencing pathways might converge in a universally conserved biochemical process that involves enzymes able to process dsRNA into small RNAs and specialized proteins like QDE2/AGO2 that use these small RNAs as guides to direct the RNA degradation. However, although in RNAi phenomena the injected dsRNA is the direct source of siRNA, in transgene-induced gene silencing additional steps are required to generate dsRNA molecules and eventually siRNA. These might involve the recognition and modification of transgenes at the DNA level. For instance, PTGS is not efficiently maintained in *Arabidopsis* mutants that are impaired in a SWI2/SNF2 chromatin component (ddm1; Jeddeloh et al. 1999) or in the major DNA methyltransferase (met1; Finnegan and Dannis 1993), suggesting that DNA methylation and chromatin structure play a key role in transgene-induced gene silencing (Morel et al. 2000). A similar role in modifications of chromatin of transgenic loci has been also hypothesized for the RecQ DNA-helicase QDE3 in *N. crassa*.

An important part of the silencing mechanism is also played by the RNA-dependent RNA polymerases (RdRP). Although the role of RdRP is still not completely clear, evidence exists that in transgene-induced gene silencing phenomena, RdRPs are required to convert transgenic RNA to dsRNA (Dalmay et al. 2000). However, a putative RdRP encoded by *ego-1* was also found to be partially required for RNAi in *C. elegans*, in which the silencing inducer is a dsRNA, suggesting that the function of RdRPs is not merely the production of an initial dsRNA from a single-stranded RNA template. Indeed, recent findings indicate that siRNAs can work as primers for RdRP activity both on dsRNA and sense-targeted mRNA templates (Lipardi et al. 2001). Thus, RdRP could work in a silencing amplification step by copying dsRNA, increasing their concentration, or converting part of the target mRNA into dsRNA. This amplification step could be important to increase the effectiveness of PTGS (Lipardi et al. 2001; Sijen et al. 2001). However, our results show that a mutation in *qde-1* completely abolishes both target mRNA degradation and siRNA accumulation, suggesting that the RdRP codified by *qde-1* function is not limited to the ancillary function of increasing silencing strength but is likely to be required for the initial step of dsRNA production using single-stranded transgenic RNAs as templates.

In addition to the role played in posttranscriptional gene silencing, the QDE2 family of proteins has been shown to have important functions in development. For example, in *Drosophila*, the *piwi* gene is necessary for the maintenance of the germ-line stem cell population (Cox at al. 1998), and *ago1* is required for embryogenesis (Kataoka et al. 2001). In *C. elegans*, two other members of this family, *alg-1* and *alg-2*, are also involved in development (Grishok et al. 2001). It is interesting to note that endogenously produced small RNAs, called small temporal RNAs (stRNAs), similar in size with siRNA are also involved in specific developmental gene regulation events in *C. elegans*. However, whereas in gene silencing siRNAs are used as guides to induce degradation of homologous mRNA, in developmental gene regulation stRNAs work as guides to block translation of target mRNA containing the 3′ untranslated region sites complementary to the stRNA. Thus, it appears that small RNAs in association with proteins belonging to the QDE2 family may be part of a general conserved mechanism to control gene expression either at the posttranscriptional or translational level. The recent identification of a number of genes coding for small RNAs in vertebrates and invertebrates (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001) further supports the argument for the widespread nature of such a regulatory mechanism. Therefore, the characterization of the function of small RNAs and the QDE2 protein family has implications not only in gene silencing but also in gene regulation in general.

Materials and methods

Plasmids and Neurospora *strains*

The 6xw stable silenced strain of *Neurospora* was obtained by transforming spheroplasts of strain FGSC no. 3958 a (*qa-2; aro-9*) with pX16 containing the *qa-2* gene as a selectable marker and a 1500-bp fragment of the coding sequence of *al-1*. The *qde* mutants used for small RNA detection were obtained by UV mutagenesis of the 6xw strain (Cogoni and Macino 1997). The 820 strain was obtained by transforming 6xw with pMXY2, which contains a benomyl-resistance β-tubulin gene that functions as a dominant selectable marker in *N. crassa*. Benomyl-resistant transformants were selected, and the orange phenotype was rescued. Southern analysis revealed the deletion of the *qde-2* locus probably as a consequence of recombination of integrated plasmids (PMYX2; data not shown).

The pal-1.6 plasmid was derived from a pBluescript II SK+ vector in which a 1500-bp *Xba*I/*Cla*I fragment of *al-1* was cloned. The pal-1.6 plasmid was digested with *Cla*I and transcribed with a T3 RNA polymerase to obtain a sense transcript (probe 1); it was then digested by *Xba*I and transcribed with a T7 RNA polymerase to obtain an antisense *al-1* fragment (probe 2). A pBluescript II SK+ was digested with *Pvu*II, transcribed with T3 RNA polymerase for the RNA molecules, and used as probe 3. A pBluescript II SK+ was digested with *Ssp*I, transcribed with T7 RNA polymerase for the RNA molecules, and used as probe 4. To produce the plasmid for Flag–QDE2 expression, a sequence containing the entire *qde-2* gene with its own promoter was cloned into the *Bam*HI site of a pBluescript II SK+ without its *Hin*dIII site in the polylinker. To

introduce the Flag epitope at the N terminus of the protein, an 80-bp fragment was removed and was substituted with a PCR homologous fragment amplified with 5′-oligo: 5′-CCCAAGCTTGACTACAAGGAC GACGATGACAAATCGCTCAGCGAGAAGGAG-3′ (containing a *HindIII* restriction site, a 24-bp sequence coding for Flag epitope, and a region of homology to *qde-2* sequence) and with 3′-oligo: 5′-CCCAAGCT TCACCTTCTCGCCCATGGT-3′.

QDE2 purification and Western analysis

Frozen mycelia were homogenized in 10% glycerol, 50 mM HEPES, and 135 mM KCl. Extracts were incubated 5 min on ice. After microcentrifugation at 4°C for 10 min, SDS-loading buffer was added to supernatants, and proteins were denatured at 94°C for 5 min. All protein buffers contained leupeptin (1 μM), pepstatin (1 μM), and phenylmethanesulfonyl fluoride (50 μM). To purify the Flag–QDE2 protein, mycelia of strains expressing the Flag–QDE2 and of the 6xw strain as a control were filtered through cheesecloth, resuspended in 140 mM KCl, 20 mM Tris-HCl (pH 8), and 5 mM $MgCl_2$, and homogenized. Total extracts were centrifuged at 4°C for 20 min, and supernatants were incubated with α-flag M2 affinity gel. After 3 h, beads were harvested and washed with resuspending buffer. Elution was done in the same buffer containing 100 μg/mL Flag peptide.

Western blot analyses were performed as follows. *Neurospora crassa* protein extracts were separated by electrophoresis on 7% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membrane. Blots were probed with α-flag monoclonal (Sigma) or α-QDE2 polyclonal antibody. Polyclonal antibodies were raised against two QDE2 peptides: amino acids 115–129 (WTEPSSNQNLPSKPQ) and amino acids 316–330 (GDERGKQKDGKEVRY). Two rabbits were immunized with a mix of the coupled peptides, and the antiserum was used for Western analysis in a dilution of 1:1000. All blots were blocked and washed in TBST with 5% nonfat dry milk, followed by horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG (Sigma, BioRad) secondary antibody incubation. The ECL Western blot chemiluminescence detection kit was applied for immunodetection (Amersham).

Small RNA purification and Northern analysis

Small RNA purification was performed as described by Hamilton and Baulcombe (1999) with minor modifications. Frozen mycelia were homogenized with a potter in 50 mM Tris-HCl (pH 9.0), 10 mM EDTA, 100 mM NaCl, and 2% SDS. The homogenates were extracted with an equal volume of phenol-chloroform, and the nucleic acids were precipitated by adding 3 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate (pH 5), over night at −20°C. After centrifugation the pellets were washed in 70% ethanol, dried, and resuspended in double distilled water. Incubating this solution for 30 min on ice with polyethylene glycol (MW 8000) at a final concentration of 5% and 500 mM NaCl, we precipitated nucleic acids with high molecular weight whereas the small RNA molecules remained in the solution. The supernatants were precipitated with ethanol as described above. The concentration of the RNA preparation was quantified by spectrophotometric analysis. Low-molecular-weight RNAs were separated by electrophoresis in 0.5× TBE through 15% polyacrylamide 7 M urea. Ethidium bromide staining was used to verify the correct loading. Then RNA was electrotransferred in 1× TBE onto Gene Screen Plus filters (New England Nuclear), and fixed by ultraviolet cross-linking. To control the size and polarity of low-molecular-weight RNAs, 25-mer oligonucleotides were used as molecular size markers. Oligonucleotides 5′-GCAAGAACGGAAAGAAGCCAAAGGC-3′ and 5′-CA TGAGCAACGTGACGACAAGGCGG-3′ were used as sense and antisense controls, respectively, in hybridizations performed with RNA probes 1 and 2; oligonucleotides 5′-CCTCGAGGGGGGGCCCGGTAC CCAA-3′ and 5′-AAAACGACGGCCAGTGAGCGCGCGT-3′ in those with RNA probes 3 and 4. Prehybridization and hybridization were at 35°C in 50% deionized formamide, 7% SDS, 250 mM NaCl, 125 mM sodium phosphate (pH 7.2), and sheared, denatured, salmon sperm DNA (100 mg/mL). After overnight hybridization, membranes were washed twice in 2× SSC and 0.2% SDS at 35°C for 30 min and once in 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 60 mM sodium chloride, and 10 μg/mL RNase A at 37°C for 1 h to remove unspecific background.

Single-stranded RNA probes were transcribed in both sense and antisense orientation by pal16 and pal17 plasmids with ^{32}P-labeled uridine triphosphate (50 μCi per 20 μL reaction volume; specific activity 3000 Ci/mmole; New England Nuclear), using T3 RNA polymerase (Roche). To remove plasmid template, the reaction was incubated at 37°C for 15 min with RNase-free DNase I (Roche). To break labeled transcripts to an average size of 50 nt, 300 μL of 80 mM sodium bicarbonate and 120 mM sodium carbonate were added to the transcriptional reaction and incubated at 60°C for 3 h. To stop the hydrolysis reaction of the transcript, 20 μL of 3 M sodium acetate (pH 5.0) was added (Hamilton and Baulcombe 1999).

Acknowledgments

We thank Emma Forrest for revising the manuscript. This work was supported in part by grants from the Istituto Pasteur Fondazione Cenci Bolognetti, from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica.

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Cellular RNA-Dependent RNA Polymerase Involved in Posttranscriptional Gene Silencing Has Two Distinct Activity Modes

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Summary

Recent genetic data suggest that proteins homologous to a plant RNA-dependent RNA polymerase (RdRP) play a central role in posttranscriptional gene silencing (PTGS) in many organisms. We show here that purified recombinant protein QDE-1, a genetic component of PTGS ("quelling") in the fungus *Neurospora crassa*, possesses RNA polymerase activity in vitro. The full-length enzyme and its enzymatically active C-terminal fragment perform two different reactions on single-stranded RNA templates, synthesizing either extensive RNA chains that form template-length duplexes or ~9–21-mer complementary RNA oligonucleotides scattered along the entire template. QDE-1 supports both de novo and primer-dependent initiation mechanisms. These results suggest that several distinct activities of cell-encoded RdRPs can be employed for efficient PTGS in vivo.

Introduction

Posttranscriptional gene silencing (PTGS), or RNA silencing, refers to a group of sequence-specific mRNA degradation mechanisms in eukaryotic cells (Baulcombe, 2002). First discovered in plants as cosuppression, PTGS, and virus-induced gene silencing, this phenomenon has been also reported for the filamentous fungus *Neurospora crassa* as quelling, and for a number of animals and protozoa as RNA interference (RNAi) (Cogoni and Macino, 1999b; Fire, 1999; Fjose et al., 2001). RNA silencing is induced and mediated by double-stranded (ds) RNA triggers homologous to the target template. It is used as a system of cell defense against viral RNAs, transposons, and, under the experimental conditions, transgenes and synthetic dsRNAs (Plasterk, 2002; Waterhouse et al., 2001). Importantly, RNA silencing can be used as an effective tool to combat viral diseases (Gitlin et al., 2002; Jacque et al., 2002; Novina et al., 2002).

Over the past several years, astonishing progress has been made in clarifying the molecular details of PTGS. One important step along this way was the observation that dsRNA molecules injected or even fed to the nematode *Caenorhabditis elegans* brought about the degradation of homologous host-encoded mRNAs (RNAi; Fire et al., 1998; Timmons and Fire, 1998). Soon it was discovered that the PTGS in plants is invariably linked with the accumulation of sequence-specific ~25 nt-long RNAs of both sense and antisense polarity (Hamilton and Baulcombe, 1999). The possible role of the short RNAs became apparent when it was found that double-stranded RNAi triggers are first cleaved by the dsRNA-specific nuclease, Dicer, into 21–23 nt-long dsRNAs with several-nucleotide-long 3′-protruding ends (Bernstein et al., 2001; Zamore et al., 2000). These, sometimes called small interfering RNAs (siRNAs), are then used to guide another ribonuclease complex, RISC, to the complementary mRNAs, ultimately causing its degradation (Elbashir et al., 2001b; Hammond et al., 2000).

Although dsRNA triggers are now accepted as a paradigm of RNA silencing, the way they are produced in the PTGS-committed cell is still unclear. They can arise as replication intermediates of RNA viruses or intramolecular hairpin-like transcripts produced from inverted repeat sequences. However, this does not explain the wide range of cases when RNA silencing is triggered by overexpression of ectopically inserted transgenes (cosuppression; Cogoni and Macino, 1999b). It has been proposed that cosuppression and related RNA silencing phenomena can be induced by aberrant ssRNAs (abRNAs) that are converted into dsRNA triggers by cellular RNA polymerases (Wassenegger and Pelissier, 1998). However, the exact nature of the abRNAs, as well as the details of their transformation into the double helix, remains elusive.

Genetic screens carried out in *N. crassa* have identified three loci essential for PTGS: *qde-1*, *qde-2*, and *qde-3* (from *quelling defective*; Cogoni and Macino, 1997). In plants and animals, the genetic repertoires of PTGS are more complex, yet often containing *qde* gene homologs (Hutvagner and Zamore, 2002; Waterhouse et al., 2001). Of these, the protein encoded by *qde-1* contains homology to the virus/viroid-induced RNA-dependent RNA polymerase (RdRP) from tomato leaves, so far the only cellular RdRP with biochemically documented RNA-synthesizing activity (Schiebel et al., 1993a, 1993b, 1998). Sequence homologs of RdRP are also found in many other organisms, and very often there is more than one RdRP-like gene per genome. Four such genes are found in *C. elegans*, three in *Dictyostelium*, and seven in *Arabidopsis* (Martens et al., 2002; Mourrain et al., 2000; Smardon et al., 2000).

Thus far, only *qde-1* of *N. crassa*, *ego-1* and *rrf-1* of *C. elegans*, *RrpA* of *Dictyostelium*, and *SGS2/SDE1* of *Arabidopsis* have been genetically linked with RNA silencing, mutations in other RdRP genes having little or no effect on PTGS phenotypes (Cogoni and Macino, 1999a; Dalmay et al., 2000; Martens et al., 2002; Mourrain et al., 2000; Sijen et al., 2001; Smardon et al., 2000). The second RdRP-like gene of *N. crassa*, *sad-1*, functions in the yet-to-be-characterized meiotic silencing pathway (Shiu et al., 2001).

Several models have been proposed for the role of RdRP in RNA silencing (Figure 1A). In the first model (model 1), RdRP is thought to replicate dsRNA templates, thus intensifying the silencing signal (Water-

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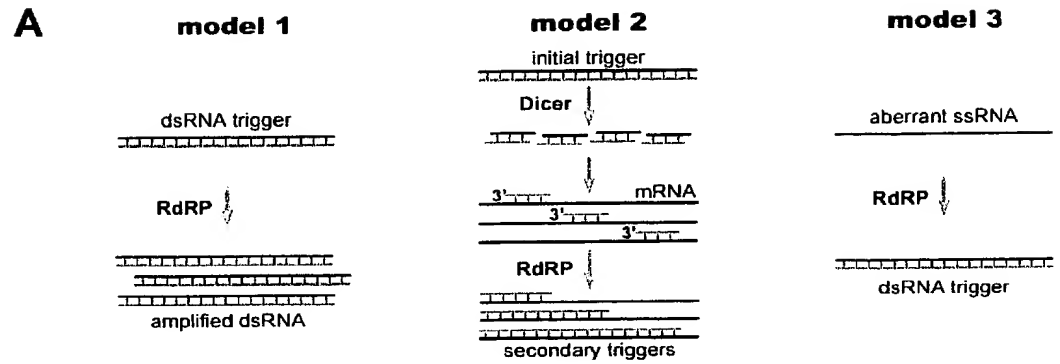

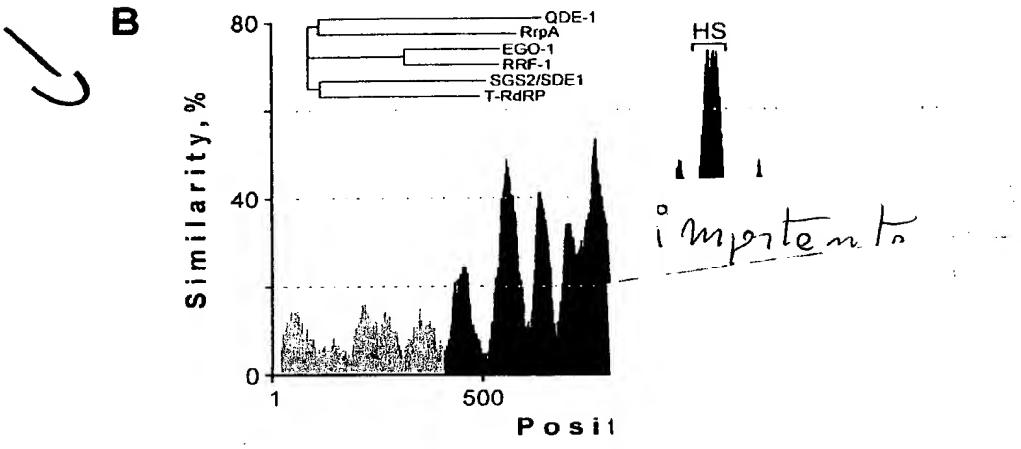

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QDE-1    (958)  VLVARSEAHFPSDIQRVRAVFKPELHSI
EGO-1    (925)  VLLTKNECIVAGDVRIFEAVDIPELHHM
RRF-1    (898)  VLLTKNECIVPGDVRIFEAVDIPELHHM
RrpA    (1307)  VMVIKNECTHPGDVRYLKAVDNIRLRHI
SGS2/SDE1 (814)  VAIAKNECLHPGDVRILEAVDVPQLHHM
T-RdRP   (752)  VVVAKNECLHPGDIRVLKAVNVRALHHM
Consensus (1013) VlvaknPcxhpgDvrileAVdxpeLhhm
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Figure 1. Cellular RdRP-like Proteins Involved in PTGS

(A) Three hypothetical models for the role of RdRP in RNA silencing. See text for details.

(B) Similarity profile for the five RdRP-like proteins with genetically documented role in PTGS and their biochemical counterpart isolated from tomato leaves (T-RdRP). The graph is generated in the AlignX program of the Vector NTI Suite using a 40 aa sliding window. The protein sequences can be accessed at http://www.ncbi.nlm.nih.gov/ under the following numbers: QDE-1, CAB42634; RrpA, CAC41974; EGO-1, AAF80367; RRF-1, AAF80368; SGS2/SDE1, AAF74208; T-RdRP, CAA71421. The 1–442 aa fragment of RrpA homologous to the Dicer helicase domain was excluded from the alignment. Regions with <20% similarity are colored gray; ≥20% similarity, black. HS refers to the most conserved span within the family of cellular RdRPs. The inset shows a phylogenetic tree for the six RdRP proteins built using the neighbor-joining method (Saitou and Nei, 1987).

(C) Protein sequence alignment for the HS span. Black shades, invariant residues; gray shades, conserved residues. The aspartate with proposed catalytic role is marked with the asterisk.

house et al., 1998). According to another model (model 2), small antisense RNAs produced by dicing the initial dsRNA trigger can be used as primers that would anneal to many mRNA templates and, after the RdRP extension step, give rise to secondary dsRNA triggers (Nishikura, 2001). Recent work on *C. elegans* has demonstrated that these secondary triggers do exist and that their synthesis requires the presence of a functional *rrf-1* gene (Sijen et al., 2001). However, the involvement of primers has not been shown in this case directly. So far the only unequivocal evidence for the primer-dependent synthesis of secondary dsRNAs comes from a study carried out in *Drosophila* embryo extracts (Lipardi et al., 2001). Interestingly, the *D. melanogaster* genome contains no homologs of the cellular RdRP family, implying that some other enzyme is responsible for the observed effects.

Finally, RdRP might be required for the synthesis of

the initial dsRNA triggers from abRNAs (model 3; Cogoni and Macino, 2000; Wassenegger and Pelissier, 1998). In line with this idea, inactivation of *qde-1* in *Neurospora* and *RrpA* in *Dictyostelium* completely abolishes both degradation of target mRNAs and the accumulation of 23–25 nt RNA species (Catalanotto et al., 2002; Martens et al., 2002). In *Arabidopsis*, *SGS2/SDE1* is required for PTGS induced by transgenes and cucumber mosaic virus (CMV) (Dalmay et al., 2000; Mourrain et al., 2000). However, this gene is redundant in the silencing mediated by, e.g., tobamoviral or potyviral infection, probably because these viruses can themselves form dsRNA replication intermediates.

The verification of the above models has been hampered by the unavailability of the RdRP-like proteins in purified active form. At the moment, it is not even certain if these components of PTGS can catalyze RNA synthesis, since the tomato RdRP prototype has never been directly linked with gene silencing. Furthermore, the attempts to produce enzymatically active recombinant RdRP from tomato have failed (Schiebel et al., 1998).

Here, we isolated soluble recombinant QDE-1 protein of *N. crassa* and its carboxy-terminal fragment spanning the region conserved across the RdRP family. The two proteins, purified to near homogeneity, catalyze RNA-dependent RNA polymerization on different ssRNA templates using either de novo or primer-dependent initiation modes. Surprisingly, two distinct types of products are synthesized during this reaction, full-length copies and 9–21-mer RNA oligonucleotides base-paired with the template. The possible significance of these two polymerization modes in RNA silencing is discussed.

Results

Sequence Analysis of Cellular RdRP-like Proteins

Amino acid sequences of tomato RdRP and cellular RdRP-like proteins involved in PTGS were aligned using the ClustalW algorithm (Thompson et al., 1994). The similarity plot built up on the alignment data demonstrates that the amino termini of these proteins are noticeably more divergent (<20% similarity) than the carboxy-terminal parts (Figure 1B). Within this conserved region, one particular span shows the highest similarity ("HS" in Figure 1B; and Figure 1C). If RdRP-like proteins indeed possess RNA-polymerizing activity, the elements crucial for this function are likely to reside within the C-terminal domain. In viral RdRPs, two conserved carboxylates located within motifs A and C catalyze the nucleotidyl transfer (Butcher et al., 2001; Hansen et al., 1997; Steitz, 1998). Based on the sequence context, the third aspartate from the GSDLDGDX block in the HS sequence appears to be identical to the catalytic aspartate from the C motif of viral RdRPs (GDD/N; Poch et al., 1989).

Expression and Purification of Soluble QDE-1

To study the biochemical properties of the RdRP-like component of PTGS, a full-length, intronless *QDE-1* gene was PCR-amplified from *N. crassa* genomic DNA and cloned into vector pYES2/CT under the *Gal1* promoter, in frame with the carboxyterminal His-tag. Sequence of the *QDE-1* insert did not differ on the amino acid level from the previously published sequence (Supplemental Figure S1 [http://www.molecule.org/cgi/content/full/10/6/1417/DC1]). The expression plasmid (pEM41) was introduced into *S. cerevisiae* INVSc1, and *QDE-1* expression was induced with galactose. As judged by the Coomassie-stained SDS-PAGE, the cells produced an ~163 kDa protein that was missing in the noninduced INVSc1(pEM41) and induced INVSc1(pYES2/CT) controls. His-tag-specific antibodies recognized the protein on Western blots, further confirming its identity (data not shown). Approximately one-third of the full-length QDE-1 could be recovered in a pure soluble form by one-step purification on a nickel-chelating column (Figure 2A).

A similar expression and purification strategy was utilized to obtain soluble truncated QDE-1 missing 376 aa from the N terminus (ΔN), as well as ΔN with the D1011A point mutation (ΔN^{DA}; numeration for the full-length QDE-1), destroying the potential catalytic aspartate (Figure 2A; and see Figure 1C). Typical yields of purified proteins were ~0.5 mg (QDE-1) or 3–5 mg (ΔN and ΔN^{DA}) per 1 liter of yeast culture. Large amounts of ΔN could be produced in *E. coli* using an appropriate expression plasmid, but all were produced as inclusion bodies despite our optimizations.

Purified QDE-1 Is Enzymatically Active In Vitro

Recombinant QDE-1 isolated from *S. cerevisiae* was assayed for RdRP activity at 30°C in a mixture containing firefly luciferase (luc) mRNA, four ribonucleoside triphosphates (NTPs), including [α-^{32}P] UTP, under the conditions optimal for several viral RdRPs (Yang et al., 2001). The presence of QDE-1 in the mixture correlated with the appearance of two distinct reaction products (Figure 2B, lanes 1 and 2), one migrating in native agarose gel slightly slower than the ssRNA template (lower band), the other one co-migrating with the dsRNA of the template length (upper band). To rule out that the products were synthesized by yeast enzymes contaminating the QDE-1 preparation, we assayed similarly purified control fractions from galactose-induced cells containing vector (pYES2/CT) or plasmid encoding His-tagged LacZ (pYES2/CT/*lacZ*; Invitrogen). No polymerization products were detected in either of the two cases (data not shown).

Observed Activity Is a Function of QDE-1 C-Terminal Domain

To characterize the newly found activity, purified soluble ΔN (carboxyterminal fragment of QDE-1) was assayed with the luc template as described above. The two labeled RNA products were apparent on the autoradiogram, similarly to the QDE-1 catalyzed reaction (Figure 2B, lane 3). Specific activity of ΔN is similar to that of QDE-1, thus implicating the C-terminal domain in the catalysis.

Notably, no activity was detected when ΔN^{DA} (ΔN with the putative catalytic residue Asp1011 changed to Ala) was assayed under the same conditions (Figure 2B, lanes 4 and 5). To make sure that the loss of activity in ΔN^{DA} is not because of an alteration in the protein fold, both ΔN and ΔN^{DA} were subjected to gel-filtration under native conditions, as depicted in Figure 2C for ΔN. There

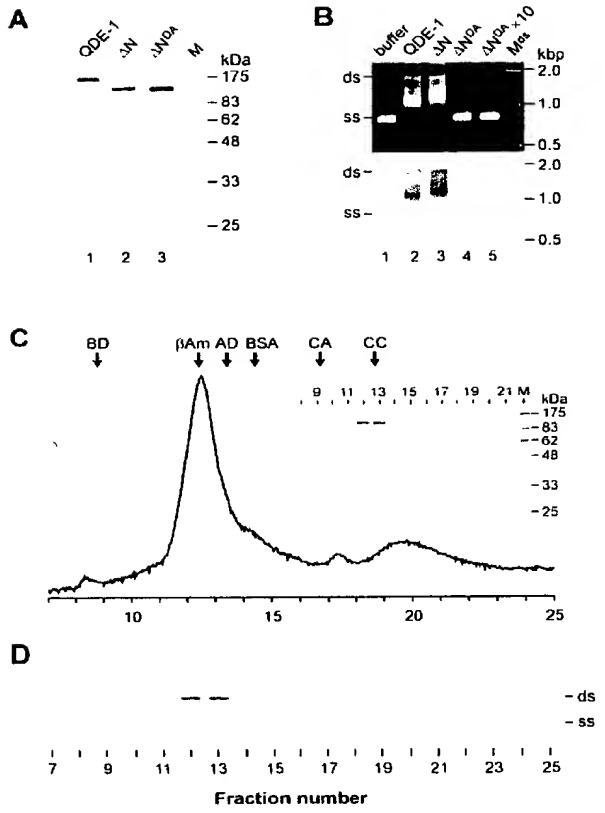

Figure 2. Purified QDE-1 and Its C-Terminal Fragment Possess RNA Polymerase Activity

(A) SDS-PAGE analysis of purified QDE-1 (lane 1), ΔN (lane 2), and ΔN^{DA} (lane 3). M, marker lane. Molecular masses of the protein standards are indicated on the right. Sequence-deduced molecular masses of His-tagged QDE-1 and ΔN (ΔN^{DA}) are 163 and 122 kDa, respectively.

(B) The purified proteins were assayed in 10 μl mixtures containing 90 μg/ml of firefly luciferase (luc) mRNA (T7 transcript of pT7luc cut with HindIII; Kolb et al., 2000) in the presence of the four unlabeled NTPs and [α-^{32}P]UTP, and the reaction products were analyzed by native agarose gel-electrophoresis. Lanes: 1, M-200 buffer control; 2, reaction containing 10 μg/ml QDE-1; 3, 10 μg/ml ΔN; 4, 10 μg/ml ΔN^{DA}; 5, 100 μg/ml ΔN^{DA}. Upper panel, ethidium bromide (EtBr) stained gel; lower panel, autoradiogram. Positions of ss and the full-length ds forms of the template RNA are shown on the left. dsDNA marker (M^{ds}) lengths are indicated on the right.

(C) A_{280} elution profile of ΔN (30 μg) separated on a Superdex 200 column (Pharmacia; 20 mM Tris-HCl [pH 8.9], 100 mM NaCl; 1 ml/min; 1 ml fractions). Arrows show the positions of gel-filtration markers (Sigma): BD, blue dextran; βAM, β-amylase; AD, alcohol dehydrogenase; BSA, bovine serum albumin; CA, carbonic anhydrase; CC, cytochrome C. Peak at 20 min corresponds to nonionic detergents from the RdRP storage buffer. Inset, SDS-PAGE analysis of the eluate fractions.

(D) RdRP activity measured in the Superdex 200 fractions using luc RNA template. Note that the activity peak corresponds to the position of ΔN protein (fractions 12–14).

was no detectable difference in the position or shape of the ΔN and ΔN^{DA} peaks, thus indicating that ΔN^{DA} is properly folded (data not shown). Position of the ΔN protein peak coincided with the peak of enzymatic activity, thus providing additional evidence against possible contamination with cellular enzymes (Figure 2D).

QDE-1 Catalyzes RNA-Dependent RNA Polymerization

To ascertain that the reaction catalyzed by QDE-1 was RNA-dependent RNA polymerization, the enzyme was assayed in the mixtures lacking either template or unlabeled nucleotides (ATP, CTP, and GTP). As expected, no labeled products were formed in these two reactions (Figure 3A, lanes 2 and 4). Purified RdRP subunit of dsRNA virus ϕ6 (ϕ6Pol), used here as a control, produced a full-length dsRNA product, which was expected from our previous work (Figure 3A, lane 1, and see Makeyev and Bamford, 2000b). Similarly to ϕ6Pol, the upper band in the QDE-1 directed reactions is likely to arise through the end-to-end polymerization. Because of their intermediate mobility between the ss and ds species, the faster migrating products can appear as a result of incomplete synthesis, with only part of the template being converted into the double-stranded form.

To rule out the possibility that the labeled RNA products were due to a phosphate transfer to the template rather than nucleotidyl transfer to the daughter strand, reactions were carried out with biotin-11-CTP where the label is tethered to the nucleotide base. Reaction products were immobilized on a membrane and probed with HRP-streptavidin conjugate (Figure 3B). A strong signal was detected for the mixtures containing both polymerase (ΔN) and the template (luc), thus indicating that the labeled cytosine was incorporated into the product fraction.

To examine whether the nucleotide composition of the RNA products was instructed by the template, we took advantage of the "nearest neighbor" analysis that allows one to determine a distribution of the nucleosides 5′-adjacent to α-labeled nucleoside-5′-monophosphates incorporated in the RNA product. QDE-1 was incubated with either luc RNA or poly(A) homopolymer in the presence of 0.2 mM of each of the four unlabeled NTPs and [α^{32}P]UTP. In both reactions, labeled products were readily detectable by agarose gel electrophoresis and TCA precipitation (data not shown). The reaction products were digested with RNase T2 to generate nucleoside-3′-monophosphates (Ap, Cp, Gp, and Up), which were separated by thin-layer chromatography (TLC). In the case of template-dependent synthesis, all four nucleoside-3′-monophosphates will carry labeled phosphate for the luc template, with the Cp, Gp, Ap, and Up distributed as 1.0 to 1.1 to 1.4 to 1.8. Only labeled Up is expected for the poly(A)-programmed reaction. Figure 3C confirms these predictions completely.

QDE-1 Can Initiate RNA Synthesis De Novo

Many RdRPs utilize primer-independent initiation mechanism (Butcher et al., 2001; Laurila et al., 2002; and references therein). To test whether QDE-1 can also support primer-independent (de novo) initiation, the polymerase was assayed in the presence of γ-labeled nucleotides. The first 5′-terminal nucleotides of daughter strands initiated de novo should retain its triphosphate group, whereas only α-phosphates will be incorporated in the case of primed synthesis. Labeled RNA products were produced both in the presence of [γ-^{32}P]GTP and [γ-^{32}P]ATP, although the incorporation efficiency of [γ-^{32}P]GTP was noticeably higher. Figure

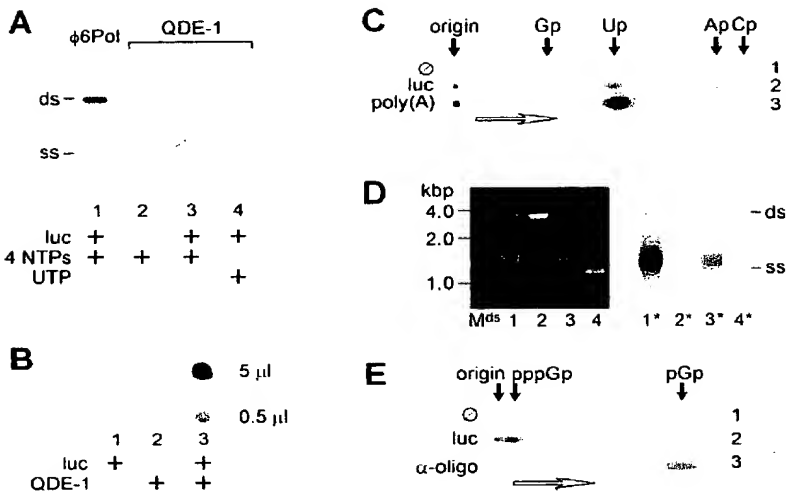

Figure 3. QDE-1 Catalyzes RNA-Dependent RNA Polymerization

(A) QDE-1 (4 μg/ml) was incubated in mixtures containing either all four NTPs (1 mM of ATP and GTP and 0.2 mM of CTP and UTP, lanes 2–3) or only 0.2 mM of UTP (lane 4) in the presence of a constant amount of [α-^{32}P]UTP. 40 μg/ml of bacteriophage φ6 RdRP (lane 1; φ6Pol) was assayed as a control in the presence of the four NTPs. In all cases except lane 2, mixtures contained 90 μg/ml luc RNA. Reaction products were separated in 1% agarose gel under native conditions. Positions of the ss and full-length ds forms of the luc RNA are shown on the left.

(B) Reaction mixtures containing 1 mM each of ATP and GTP, 0.2 mM of UTP, and 0.2 mM of biotin-11-CTP (NEN) were incubated for 1 hr at 30°C. RNA products were immobilized on a Hybond N+ membrane (Amersham Biosciences), and the membrane was stained with HRP-conjugated streptavidin (NEN) and developed using an ECL detection kit (Pierce). 1, reaction containing 90 μg/ml luc RNA; 2, reaction containing 25 μg/ml ΔN polymerase; 3, reaction containing both ΔN and luc RNA. Upper row, 5 μl; lower row, 0.5 μl of the original reaction mixtures.

(C) RdRP reactions containing 10 μg/ml of QDE-1, 0.2 mM each of the four unlabeled NTPs, and [α-^{32}P]UTP were programmed with either 90 μg/ml luc RNA (track 2), 150 μg/ml poly(A) homopolymer (Sigma; track 3), or no RNA (track 1). Reaction products were purified from unincorporated nucleotides by gel-filtration and subjected to RNase T2 digestion and TLC separation as described (Schiebel et al., 1993a). Black arrows indicate the position of the four nucleoside-3′-monophosphates. The open arrow shows the TLC developing direction.

(D) EtBr-stained agarose gel (lanes 1–4) and corresponding autoradiogram (1*–4*) for RdRP reactions carried out with 100 μg/ml 5′Δm$_6^+$ RNA in the presence of [γ-^{32}P]GTP (lanes 1, 2, 4 and 1*, 2*, 4*) or [γ-^{32}P]ATP (lane 3 and 3*). Lanes: 1 (1*) and 3 (3*), 20 μg/ml ΔN polymerase; 2 (2*), 20 μg/ml φ6Pol; 4, M-200 buffer. Other designations as in Figure 2B.

(E) Reactions done as in (D) were purified from unincorporated nucleotides, treated with RNase T1 and separated by TLC. Tracks: 1, no RNA; 2, luc RNA; 3, a control RNA oligonucleotide with the 5′ terminal G labeled with T4 PNK and [γ-^{32}P]ATP. Black arrows, positions of the released nucleotides; open arrow, TLC developing direction.

3D shows that using the 5′Δm$_6^+$ RNA template (a φ6-specific RNA with the ...UUCC-3′ terminus; Makeyev and Bamford, 2000a) QDE-1 incorporated the label predominantly into the lower band (partially double-stranded species), while the upper band (full-length dsRNA) was labeled very weakly. A similar pattern was observed for the luc template (data not shown). This indicates that the partial dsRNA species might contain multiple copies of de novo-initiated daughter strands annealed to the template.

To exclude that the γ-phosphate was introduced through a γ-phosphate transfer rather than RNA polymerization, we treated labeled RNAs with the guanosyl-specific RNase T1 and analyzed the digest by TLC (Vasilieva et al., 2000). The label was released from the QDE-1 RNA products as pppGp (Figure 3E). In the control digestion, containing the oligonucleotide 5′-GUUUUCACC CUAUCCUCCCC-3′ labeled at the 5′ α-position with T4 polynucleotide kinase, the label was retrieved in the form of pGp, as expected.

QDE-1 Accepts a Number of ssRNA Templates

We further assayed QDE-1 with a number of single-stranded templates, such as green fluorescent protein (GFP) mRNA, genomic RNA of tobacco mosaic virus (TMV), as well as several *N. crassa* and φ6-specific RNAs (Figure 4A and Supplemental Figure S2A [http://www.molecule.org/cgi/content/full/10/6/1417/DC1]). In all cases, QDE-1 produced full-length dsRNA and the partially double-stranded species, exactly as for the luc template. In some experiments, when reaction products were purified before electrophoresis by gel-filtration on Sephadex G-50 equilibrated with water, bands migrating as ~20 nt ssRNA were also apparent on the autoradiograms (arrowhead in Figure 4A). Neither these nor the partially dsRNA products were present in the corresponding φ6Pol lanes, thus suggesting that they might be a specific trait of the cellular RdRP involved in the RNA silencing process.

The Nature of the Reaction Products

To identify the nature of the QDE-1 polymerization products, two independent approaches were employed. First, RdRP products were separated by electrophoresis in a formaldehyde-containing gel (Figure 4B). Under these conditions, the RNA strands of dsRNA products are completely denatured and migrate according to their length. Three bands were detected for the luc mRNA-programmed reaction: a faint band of the template length (1×), a more intense band twice as long as the template (2×), and the strongest band migrating at the ~20 nt position (sRNA, for brevity). φ6Pol only produced the 1× and 2× products, with no signs of the sRNA. It has been shown earlier that the 1× species arises as a result of de novo-initiated RNA synthesis, whereas the 2× originates by extending the folded back 3′ end of the template (so-called "back-priming;" Laurila et al., 2002). Thus, QDE-1 predominantly used the back-priming mode to produce the complete or nearly complete copy of the luc mRNA. On the native gel, this product migrates as the template-length dsRNA (see Figure 4A). The sRNA products are apparently derived from the incomplete dsRNA product. Since this product migrates noticeably slower than the luc mRNA on the native gel,

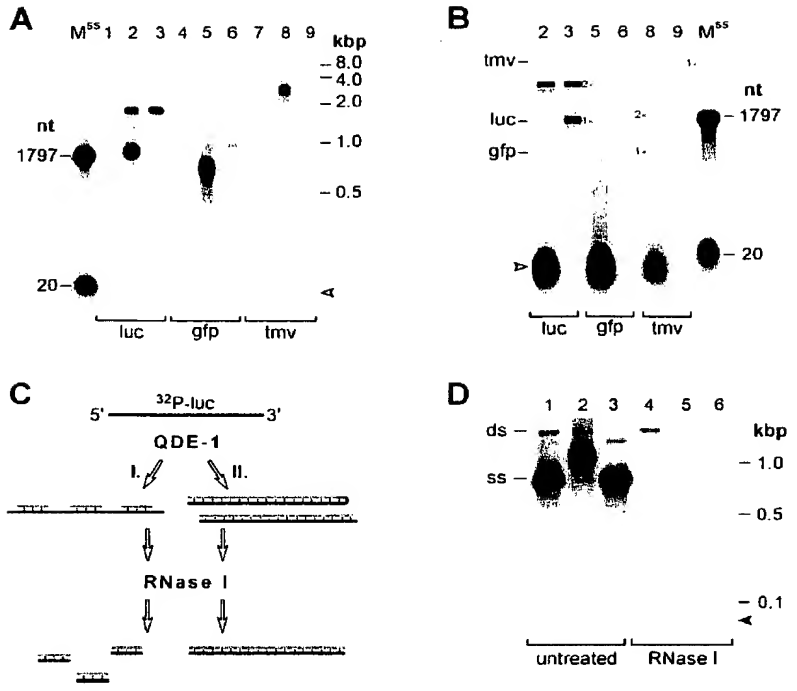

Figure 4. QDE-1 Generates Two Types of Reaction Products

(A–B) RdRP reactions were programmed with 90 μg/ml luciferase mRNA (lanes 1–3), 80 μg/ml of GFP mRNA (lanes 4–6; T7 transcript of plasmid #TU58 [Chalfie et al., 1994] cut with EcoRI), or 100 μg/ml of TMV genomic RNA (lanes 7–9). Reactions contained [α-^{32}P]UTP and either 10 μg/ml QDE-1 (lanes 2, 5, 8) or 20 μg/ml φ6Pol (lanes 3, 6, 9). Lanes 1, 4, 7 are "buffer only" controls. (A) native 1.2% agarose gel. Small RNA products (sRNAs) are indicated with the open arrowhead. dsDNA marker positions are shown on the right. (B) formaldehyde-containing 1.5% agarose gel. Positions of the single-stranded templates are shown on the left. M^{ss}, ^{32}P-labeled ssRNA markers (20 and 1797 nt). (C) Schematic and (D) actual results of the RNase protection assay. RdRP reactions were carried out with 70 μg/ml of ^{32}P-labeled luc mRNA and no labeled NTPs. Reactions contained 40 μg/ml φ6Pol (lanes 1 and 4), 40 μg/ml QDE-1 (lanes 2 and 5), or M-200 buffer (lanes 3 and 6). On 1 hr incubation at 30°C, polymerization was stopped by EDTA and aliquots were incubated with RNase I (lanes 4–6) or RNase I reaction buffer (lanes 1–3), as specified under Experimental Procedures. The black arrowhead indicates the position of short dsRNA fragments in lane 5. The faint slow-migrating band in lane 3 apparently represents a conformer of luc mRNA.

multiple copies of complementary sRNA are likely produced on each template molecule. It is obvious from Figure 4B that QDE-1 employs a predominantly de novo initiation mechanism to produce small amounts of nearly full-length dsRNA on the GFP and TMV templates. And again, complementary sRNAs migrating in the 20 nt region represent the major reaction product.

For the second approach, we used the property of RNase I to degrade ssRNA but not dsRNA at a high ionic strength. If QDE-1 produces two discrete types of dsRNA elements, as anticipated from the above experiments, two products will withstand RNase I digestion: (1) short dsRNAs and (2) (nearly) full-length dsRNA (Figure 4C). Both RNase-resistant species were indeed detected after the electrophoretic separation on a native agarose gel (Figure 4D), thus supporting our conclusions.

sRNAs Are 9–21 nt Long

To accurately determine the lengths of the sRNAs, we carried out polymerization reactions with different RNA templates in the presence of γ-labeled GTP. After the incubation at 30°C, reaction products were analyzed using a high-resolution urea-containing PAGE. Regardless of the template, sRNAs appeared as a population of 9–21-mer oligonucleotides with occasional week bands of shorter and longer products (Figure 5 and Supplemental Figure S2B [http://www.molecule.org/cgi/content/full/10/6/1417/DC1]). The sRNA patterns by QDE-1 and ΔN were identical; no sRNA was detected in the φ6Pol controls (Figure 5). Similar patterns were obtained when α-labeled UTP was used instead of γ-label, with the only difference being that the relative intensity of longer products was higher (data not shown).

sRNAs Are Evenly Distributed along the Template

It was not obvious from the above experiments whether the complementary sRNAs are clustered at some particular region of the template RNA (e.g., close to 3′ or 5′ termini) or distributed evenly. To distinguish between these two possibilities, γ-labeled sRNAs synthesized on the luc mRNA were used as probes for Northern blotting (Figure 6A). Six RNAs were used as the hybridization targets: four sense fragments of luc RNA spanning different regions as shown in Figure 6B, full-length antisense luc RNA (a-luc), and a control sR5 RNA originating from the φ6 s$^+$ RNA and containing no homology to the luciferase gene. All four sense RNAs were recognized by the probe, thus suggesting that the sRNA population contains species complementary to the different template segments (Figure 6C). As expected, no signal was detected in the sR5 lane. There was a very weak labeling of the a-luc RNA band, which could be formally explained by the presence of low amounts of sense sRNAs in the probe. However, this might also be an artifact of hybridization, since low stringency conditions were used.

Reactions with Primed ssRNA and Blunt-Ended dsRNA Templates

Models 1 and 2 in Figure 1A imply that QDE-1 can utilize dsRNA templates and/or extend complementary primers annealed to a ssRNA template. To address these predictions, we assayed QDE-1 and ΔN with blunt-

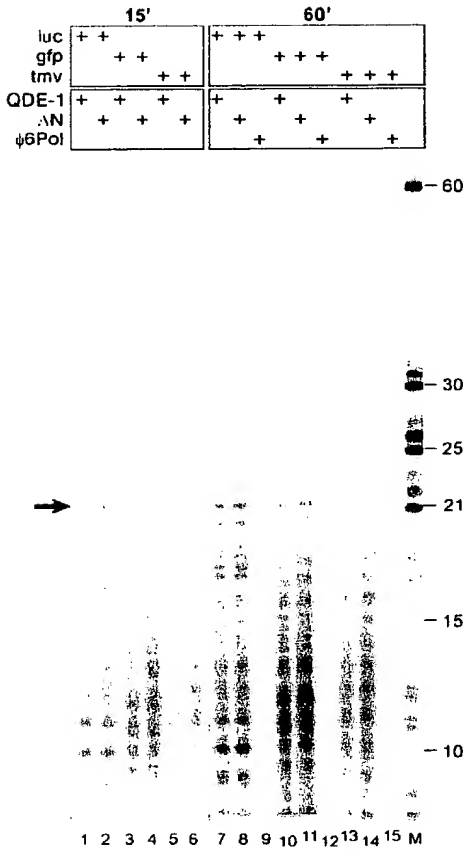

Figure 5. Size Distribution of sRNAs

Reactions were carried out as in Figures 4A–4B but in the presence of [γ-^{32}P]GTP and the additives indicated on the top of the panel. Aliquots were withdrawn at 15 min (lanes 1–6) and 60 min (lanes 7–15) time points and analyzed by 15% urea-containing PAGE. M, ^{32}P-labeled ssRNA markers produced by T7 transcription of a mixture of DNA templates. Marker positions are indicated on the right (in nt). The arrow indicates the position of 21-mer RNAs. Only the lower half of the gel is presented.

ended dsRNAs extracted from ϕ6 or yeast L-A virus (Figure 7A). No labeled products were detected even after prolonged exposures. In contrast, ϕ6Pol produced readily detectable dsRNA-labeled products that were synthesized via a semi-conservative (strand-displacement) mechanism, as expected (Makeyev and Bamford, 2000a).

To assess the primer extension capacity of QDE-1, sR5 RNA annealed with a labeled complementary RNA 20-mer was added to the QDE-1 reaction mixture containing no labeled NTPs, as illustrated in Figure 7B. The reaction was incubated at 30°C for 1 hr and analyzed by urea-containing PAGE (Figure 7C). A detectable amount of the full-length extended product was detected in the QDE-1 lane, with no band at this position in the "buffer only" control. A similar product was also visible in the ϕ6Pol lane. Under the conditions employed, reverse transcriptase of avian myeloblastosis virus (AMV-RT) produced 10–20 times more of the extended product (cDNA) than either of the two RdRPs.

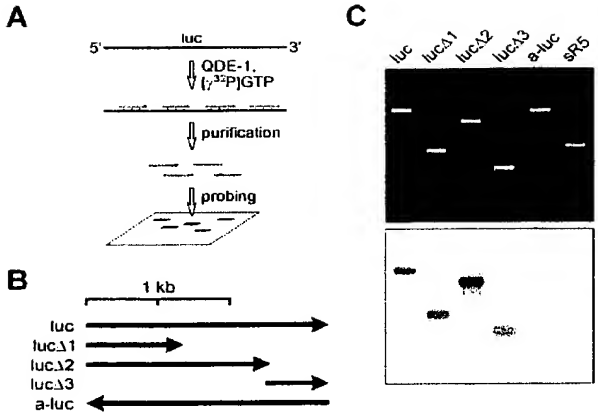

Figure 6. sRNAs Are Synthesized along the Entire Template Length

(A) sRNAs were synthesized with QDE-1 on luc mRNA, purified through agarose gel, and used to probe immobilized target RNAs.

(B) Diagram shows luciferase-specific target RNAs: luc, lucΔ1 (T7 transcript of pTZluc(-stop) cut with EcoRI; Makeyev et al., 1996), lucΔ2 (T7 transcript of pTZluc(-stop) cut with EcoRV), lucΔ3 (T7 transcript of pEM54 cut with XhoI), and a-luc (T7 transcript of pGEMluc cut with BamHI; Promega).

(C) Target RNAs as in (B) and ϕ6-specific sR5 RNA (T7 transcript of pLM659 cut with EcoRV; Gottlieb et al., 1992) were separated by formaldehyde-containing 1.5% agarose gel electrophoresis, transferred to a membrane and probed with the luc-specific sRNAs. Upper panel, EtdBr-stained gel; lower panel, autoradiogram of the membrane, after hybridization.

Discussion

Two major groups of RNA-dependent RNA polymerases (RdRPs) are known, those encoded by RNA viruses and

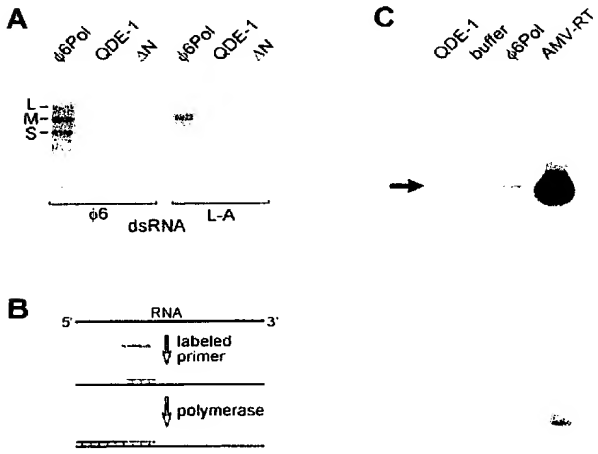

Figure 7. Reactions with dsRNA and Primed ssRNA Substrates

(A) RdRP mixtures containing 200 μg/ml of ϕ6 genomic dsRNAs (segments L, M, and S) or 100 μg/ml L-A virus genomic dsRNA were incubated with 40 μg/ml QDE-1, ΔN, or ϕ6Pol and analyzed by 1% native agarose gel electrophoresis.

(B) Schematic and (C) actual results of primer extension carried out with 40 μg/ml of QDE-1 or ϕ6Pol, or 500 units/ml of AMV-RT, as described in Experimental Procedures. The arrow indicates the position of the 136 nt extended product.

those of cellular origin. While many viral RdRPs have been characterized in remarkable detail, very little is known about cell-encoded RdRPs. Only in a single instance (tomato) was cellular RdRP purified to near homogeneity from viroid-infected leaves and shown to catalyze RNA synthesis (Schiebel et al., 1993a, 1993b). However, the recombinant enzyme produced in bacteria was inactive (Schiebel et al., 1998). It is not clear whether the tomato RdRP is involved in PTGS or if it plays some other role in the virus-infected cell.

Using QDE-1 of *N. crassa* as a model, this work provides the first unequivocal evidence that a genetic component of PTGS, homologous to the tomato RdRP, possesses RNA-dependent RNA polymerization activity in the absence of other cellular factors. It is notable in this respect that yeast *S. cerevisiae*, used to produce QDE-1, is devoid of RNA silencing machinery. Because many *S. cerevisiae* strains contain RNA viruses that encode their own RdRPs, a substantial effort was made to rule out possible contamination with these activities (see Figure 2).

The polymerase activity of QDE-1 resides in the C-terminal domain, in line with the similarity profile for the RdRP family (see Figures 1B and 2). Since dsRNA substrates are not recognized by QDE-1 in vitro (Figure 7A), it is very unlikely that cellular RdRPs amplify dsRNA silencing triggers as proposed in model 1 (Figure 1A). Further, QDE-1-catalyzed primer extension in vitro is inefficient as compared with, e.g., reverse transcriptase (Figure 7C), which may indicate that the primer extension is not a major function of QDE-1 in vivo. Interestingly, most known RNA polymerases rely on a de novo initiation mechanism (e.g., Butcher et al., 2001; Laurila et al., 2002) with only few exceptions where short oligonucleotides or proteins are used as primers (e.g., in some negative RNA viruses and picornaviruses; Fields et al., 1996).

This contrasts with the primed synthesis of secondary dsRNA triggers described for *Drosophila* (Lipardi et al., 2001; model 2 in Figure 1A). While the source of the primer-dependent RdRP in *Drosophila* extracts still remains to be identified, recent studies on *Drosophila* and mammalian cells cast serious doubt on the significance of RdRP activity for RNAi in these organisms (Chiu and Rana, 2002; Martinez et al., 2002; Schwarz et al., 2002). Thus, mechanisms of RNA silencing in insects and mammals may differ from those in fungi, plants, and worms.

Isolated QDE-1 readily accepts a number of ssRNA templates in vitro, producing two distinct types of RNA products: (1) full-length copies and (2) short RNA oligonucleotides complementary to the template. To produce the first product type, both de novo initiation and back-priming mode are utilized depending on the template (Figure 4B and Supplemental Figure S3C [http://www.molecule.org/cgi/content/full/10/6/1417/DC1]). In the case of de novo initiation, QDE-1 begins polymerization preferably at the 3′-most nucleotide of the template (Supplemental Figure S3). Regardless of the initiation mechanism, the long dsRNAs can be degraded by a Dicer-like ribonuclease into 21–25 nt long siRNAs as predicted by model 3 (Figure 1A). Notably, Cogoni and coworkers have recently discovered sequence-specific ~25 nt RNAs of both sense and antisense polarity in silenced *Neurospora* strains (Catalanotto et al., 2002). The appearance of these elements required the presence of functional *qde-1* and *qde-3* genes, whereas *qde-2* was dispensable.

It is a surprising observation that QDE-1 can initiate de novo RNA synthesis internally producing ~9–21 nt copies of input ssRNA (sRNAs; Figure 5 and Supplemental Figure S2B [http://www.molecule.org/cgi/content/full/10/6/1417/DC1]). This reaction is considerably more efficient than the synthesis of the full-length dsRNA (e.g., Figure 3D). It is therefore sensible to propose that QDE-1 may utilize this reaction mode in vivo. If so, at least a subset of the QDE-1 reaction products, close to 19–21 bp, would provide an ideal target for a RISC-like nuclease complex, thus inducing a localized mRNA cleavage (Elbashir et al., 2001a, 2001b; Sharp, 2001). Thus, QDE-1 may be needed for the synthesis of long dsRNA triggers and/or production of small guide RNAs. Additional studies will address the significance and possible roles of the two polymerization modes of QDE-1 in PTGS.

Further research will be also required to understand the mechanism that commands cellular RdRP to terminate on the completion of one to two turns of dsRNA. This could be similar to the RNA primer synthesis by DNA primases. A model has been suggested where the length of the primer is defined by the capacity of the primase active site (Frick and Richardson, 2001). In addition, the sRNA length distribution can be affected by RNA secondary structure in the way that more compact RNA templates give rise to shorter sRNAs (Supplemental Figure S4 [http://www.molecule.org/cgi/content/full/10/6/1417/DC1]). This may indicate that QDE-1 produces sRNAs by copying RNA regions that are free of stable secondary structure.

Experimental Procedures

Construction of QDE-1, ΔN, and ΔN^{DA} Expressing Plasmids

To construct *S. cerevisiae* expression plasmid pEM41 encoding His-tagged full-length QDE-1, wild-type *QDE-1* gene was PCR-amplified from genomic DNA of *N. crassa* DSM 1257 (FGSC 987) using a mixture of Turbo *Pfu* (Stratagene) and Taq (Promega) DNA polymerases and the primers 5′-GCCAAGCTTCCATGAACCCTATTACTC CTA-3′ and 5′-CCGAATTCATAATCGCCATTCCCTGTGA-3. The PCR fragment was inserted into the vector pYES2/CT (Invitrogen) at the HindIII-EcoRI sites. Three plasmids encoding ΔN (QDE-1 missing 1–376 aa) were constructed: pEM42, pEM46, and pEM55. For the *E. coli* expression plasmid pEM42, the 3′-terminal part of *QDE-1* gene was amplified from pEM41 using the primers 5′-GCTCAAATC CCATGGCTCGGAGCGAAGAAA-3′ and 5′-CCGAATTCTAATAATC GCCATTCCCTGTGA-3′ and inserted into pET21d (Novagen) at NcoI-EcoRI. Plasmid pEM46 initially used to produce ΔN in *S. cerevisiae* was generated by substituting the *QDE-1* HindIII-Eco81I fragment of pEM41 with the NcoI-Eco81I fragment of pEM42, the NcoI and HindIII cut termini being filled-in with the Klenow enzyme. However, better ΔN yields were obtained using pEM55. To construct this plasmid, a *QDE-1* PCR fragment produced using primers 5′-GCAAGCTTAAAATGGCTCGGAGCGAAGAAA-3′ and CTGAACTGT CGTTGCAAATCGTT-3′ was cut with HindIII-BstEII and ligated with the large fragment of the similarly cut pEM41. Plasmid pEM56 encoding ΔN with the point mutation D1011A (ΔN^{DA}) was derived from pEM55 using a QuickChange mutagenesis kit (Stratagene) and the oligonucleotides 5′-GTGGAGACTACGACGGCGCCATGGCCTGGG TCTGCTG-3′ and 5′-CAGCAGACCCAGGCCATGGCGCCGTCGTA GTCTCCAC-3′.

Expression and Purification of Recombinant Polymerases

Purified recombinant ϕ6Pol was obtained as reported earlier (Makeyev and Bamford, 2000b). To produce QDE-1, ΔN and ΔN^{DA}, plas-

mids pEM41, pEM46, pEM55, and pEM56 were introduced into *S. cerevisiae* strain INVSc1 (*his3Δ1/his3Δ1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52*; Invitrogen). The expression was done as recommended by Invitrogen. In brief, 40 ml cultures were grown overnight at 30°C in the $SC^{-Ura,+Glu}$ minimal medium at 240 rpm until OD_{600} ~3. The cells were collected by centrifugation for 5 min at 1500 g, room temperature, washed once with 20 ml $SC^{-Ura,+Raf}$, and resuspended in 300 ml of $SC^{-Ura,+Gal,+Raf}$ to a final OD_{600} of 0.4. The shaking was continued at 28°C for 22 hr. The cells were then harvested by centrifugation for 5 min at 5000 g, 4°C, washed with 100 ml of ice-cold water, and resuspended in 15 ml of ice-cold buffer H-5 (50 mM Tris-HCl [pH 9.3], 1 M NaCl, 1% Triton X-100, 1% Tween 20, 5% glycerol, and 5 mM imidazole) containing Complete Mini EDTA-free protease inhibitor cocktail (Roche; one tablet per 7.5 ml). The suspension was stored at −80°C in 5 ml aliquots until needed. Protein purification was done at 4°C. Thawed cell suspension (5 ml) was passed twice through a precooled French pressure cell at ~20,000 psi. PMSF was added to 1 mM after the first passage. The lysate was centrifuged at 13,000 g for 15 min, and the supernatant was loaded onto a 1 ml Ni-NTA column (Qiagen) equilibrated with buffer M-5 (50 mM Tris-HCl [pH 8.9], 300 mM NaCl, 0.5% Triton X-100, 0.5% Tween 20, and 5 mM imidazole). The column was washed with 20 ml of M-5 and 10 ml of M-50 (50 mM Tris-HCl [pH 8.9], 300 mM NaCl, 0.5% Triton X-100, 0.5% Tween 20, and 50 mM imidazole). Recombinant proteins were eluted from the column with M-200 (50 mM Tris-HCl [pH 8.9], 300 mM NaCl, 0.5% Triton X-100, 0.5% Tween 20, and 200 mM imidazole). Fractions were analyzed by 12.5% SDS-PAGE, and the protein concentration was determined by comparing the QDE-1 and ΔN bands with bands containing known amounts of bovine serum albumin (BSA). Purified proteins were stored on ice for 1–2 weeks without detectable loss of specific activity. For the negative controls, we used INVSc1 cells containing pYES2/CT and pYES2/CT/*lacZ* plasmids (Invitrogen). ΔN was produced also in the *E. coli* BL21(DE3) strain transformed with pEM42 essentially as described for φ6Pol.

RNA Templates

Synthetic ssRNA substrates were prepared by in vitro run-off transcription with T7 and T3 RNA polymerases in principle as described (Makeyev et al., 1996). References for the plasmids used for this purpose are given in figure legends. Plasmid pEM54 was derived from pTZluc(-stop) (Makeyev et al., 1996) by deleting the HindIII-EcoRV 5′-terminal fragment of the luciferase gene. Viral RNAs were extracted from purified virus particles (φ6, L-A, and TMV) with phenol and chloroform, precipitated with ethanol, and dissolved in water or 10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA. RNA concentration was measured by OD_{260}, and the quality was determined by electrophoresis in standard and/or formaldehyde-containing agarose gels (Sambrook and Russell, 2001).

RdRP Activity Assays

QDE-1 and its genetic derivatives were typically assayed in 10 μl reaction mixtures containing 50 mM HEPES-KOH [pH 7.8], 20 mM ammonium acetate (NH_4OAc), 6% (w/v) PEG4000, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM each of ATP and GTP, 0.2 mM each of CTP and UTP, and 0.8 unit/μl RNasin. Similar conditions were used for φ6Pol, but the reactions were usually supplemented with 2 mM $MnCl_2$, which is known to stimulate the activity of this polymerase (Makeyev and Bamford, 2000b). The final concentration of RNA substrates ranged from 50 to 250 μg/ml. Unless indicated otherwise, the mixture contained 0.1–0.2 mCi/ml of [α-^{32}P]UTP (~3000 Ci/mmol) or 0.5–1.0 mCi/ml of [γ-^{32}P] GTP or ATP (>5000 Ci/mmol; Amersham Biosciences). Reactions were initiated by the addition of a polymerase preparation to a final concentration of 4–40 μg/ml followed by incubation at 30°C for 1 hr. Reaction products were analyzed by agarose or urea-containing polyacrylamide gel electrophoreses (Makeyev and Bamford, 2000b; Sambrook and Russell, 2001) followed by autoradiography and/or phosphoimaging (Fuji BAS1500). In some cases, reaction products were purified before electrophoreses using AutoSeq G-50 spin columns (Amersham Biosciences) or ethanol precipitation.

RNase Protection Assay

Polymerase reactions were quenched by adding 250 mM NH_4OAc, 10 mM EDTA. The mixtures were then supplemented with 0.05 unit/μl of RNase I (RNase ONE; Promega) or an equal volume of RNase ONE 1×buffer, and incubated for 1 hr at 30°C. The reactions were stopped by the addition of 0.2% SDS, and the products were analyzed by standard agarose gel electrophoresis.

Primer Extension Assay

Primer extension was done essentially as described (Laurila et al., 2002). In brief, synthetic RNA oligonucleotide 5′-CGACUCAUGGAC CUUGGGAG-3′ was labeled with T4-PNK and [γ-^{32}P]ATP, annealed with sR5 RNA template, and assayed in the polymerase reaction mixtures containing 40 μg/ml of QDE-1 and no labeled nucleotide. As a control, the same primer-template substrate was incubated for 1 hr at 37°C in 10 μl mixtures containing 5 units of AMV-RT (Sigma), 8 units of RNasin, and 0.5 mM each of the four deoxynucleotide triphosphates in the recommended buffer. The reaction products were separated by 6% PAGE containing 7.5 M urea.

Northern Blot Analysis

To prepare an RNA probe, luciferase mRNA was used as a template for QDE-1-catalyzed RNA synthesis in the presence of [γ-^{32}P]GTP as outlined above. RNA products were denatured and separated using gel-electrophoresis in a low melting point agarose gel. The zone containing labeled sRNAs was excised from the gel. The sRNAs were recovered by melting the agarose at 70°C and used for probing target RNAs without further purification. Target RNAs were separated in formaldehyde-containing gels and transferred to Hybond-N+ (Amersham Biosciences) as described (Sambrook and Russell, 2001). The membranes were blocked in 6×SSC, 7%SDS for 4 hr at 68°C, which was followed by overnight hybridization at 42°C in the same buffer containing the sRNA probe (~10^5 cpm/ml). After hybridization, the membranes were washed three times with 2×SSC at room temperature followed by two washes with 2×SSC, 0.1% SDS at 42°C (30 min each wash). The membranes were air-dried and analyzed with a phosphoimager.

Acknowledgments

Drs. Sarah Butcher and Roman Tuma are thanked for critical reading of the manuscript. We are grateful to Riitta Tarkiainen and Marja-Leena Perälä for technical assistance. This work was supported by the Academy of Finland ("Finnish Centre of Excellence Program 2000-2005," grants 164298 and 172621).

Received: May 29, 2002
Revised: October 11, 2002

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Published online April 16, 2004

Nucleic Acids Research, 2004, Vol. 32, No. 7 2123–2128
DOI: 10.1093/nar/gkh530

The RNA-dependent RNA polymerase, QDE-1, is a rate-limiting factor in post-transcriptional gene silencing in *Neurospora crassa*

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Received February 12, 2004; Revised and Accepted March 17, 2004

ABSTRACT

The RNA-dependent RNA polymerase (RdRP) *qde-1* is an essential component of post-transcriptional gene silencing (PTGS), termed 'quelling' in the fungus *Neurospora crassa*. Here we show that the overexpression of QDE-1 results in a dramatic increase in the efficiency of quelling, with a concomitant net increase in the quantity of *al-1* siRNAs. Moreover, in overexpressed strains there is a significant reduction in the number of transgenes required to induce quelling, and an increase in the phenotypic stability despite progressive loss of tandemly repeated transgenes, which normally determines reversion of a silenced phenotype to wild type. These data suggest that the activation and maintenance of silencing in *Neurospora* appear to rely both on the cellular amount of QDE-1 and the amount of transgenic copies producing RNA molecules that act as a substrate for the RdRP, implicating QDE-1 as a rate-limiting factor in PTGS.

INTRODUCTION

Post-transcriptional gene silencing (PTGS) mechanisms are highly conserved: quelling in fungi, co-suppression in plants and RNA interference (RNAi) in animals all occur due to the presence of foreign nucleic acid sequences such as transgenes, transposons and viral RNAs or double-stranded RNA (dsRNA) (1). The silencing mechanism is based on the cleavage of dsRNA by dicer (2,3), producing small interfering 21–25 nucleotide (nt) RNAs (siRNA) which, in conjunction with the RNA-induced silencing complex (RISC) (4), degrade all homologous mRNAs. While dsRNA has been shown to trigger PTGS directly (5), it is still unclear how dsRNA originates in transgene-induced silencing. Current models (6,7) propose that aberrant single-stranded transgenic transcripts (abRNA) are converted to dsRNA by a cellular RNA-dependent RNA polymerase (RdRP). The identification of the quelling-defective gene *qde-1* in *Neurospora* was the first experimental evidence of the involvement of an RdRP in PTGS (8). QDE-1 RdRP activity *in vitro* was shown to catalyse *de novo* or primer-independent RNA polymerization on a single-stranded RNA (ssRNA) template (9). As well as initiating the transgene-induced silencing mechanism, RdRP may also be responsible for the amplification and maintenance of the silencing signal by synthesis of secondary dsRNA trigger molecules, which in turn would be processed into secondary siRNAs.

In general, the introduction or the direct expression of dsRNA in a eukaryotic cell is sufficient to initiate silencing. When transgenes are introduced in plants and fungi, however, this sequence-specific degradation does not always occur. As the mere presence of transgenic DNA, although necessary, is not sufficient to activate silencing, it has been proposed that only particular transgenic loci are able to work as silencing inducers. In plants for instance, highly expressed transgenes are better inducers than poorly expressed ones (10), suggesting that transgenic mRNAs may only trigger silencing when they exceed a given threshold. Moreover, a correlation between the presence of tandemly arranged transgenic loci and the occurrence of silencing has been observed in both plants and fungi (11,12). It has therefore been suggested that tandem transgenic repeats are good inducers of silencing, even without a high rate of transcription, because they produce RNAs that are somehow 'aberrant', which are specifically used as substrates for RdRPs. However, the presence of a tandem repeat per se is not sufficient to elicit a silencing response (13,14). Furthermore, it is still unclear whether the correlation between tandem repeats or highly expressed transgenes and the activation of silencing reflects special features of such transgenes, or suggests the existence of a threshold for either transgenic RNAs or transgenic copy number above which cells activate the silencing machinery. A simple model would be that transgenes or duplicated DNA are able to produce a silencing trigger (i.e. an RNA substrate for RdRP) per se, and only when such a trigger reaches a given threshold is silencing efficiently elicited.

In order to gain insight into the initiation of transgene-induced gene silencing, we decided to overexpress the RdRP *qde-1* in *Neurospora crassa*. This was based on the hypothesis that augmenting the level of QDE-1 may increase conversion of transgenic RNA into dsRNA, leading to the activation of silencing in all transgenic strains, including those that supposedly express the RNA trigger below a given threshold. In this paper we present data showing that the overexpression

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of QDE-1 leads to a dramatic increase in quelling efficiency (i.e. percentage of transgenic strains showing silencing). Moreover, in overexpressed strains there is a significant reduction in the number of transgenes required to induce quelling, and a concomitant increase in phenotypic stability. Thus, the activation and maintenance of silencing in *Neurospora* appears to rely on the relative concentration of both QDE-1 and its transgenic RNA substrates.

Figure 1. Northern analysis of wild-type strains transformed with the *qde-1* overexpression cassette. Overexpression of *qde-1*, induced by the addition of quinic acid, was revealed in transformants 3 and 6. Both transformants showed the same silencing efficiency (data not shown). Subsequent experiments were carried out with transformant 6 as the *qde-1*-overexpressing strain OQ1. The shadow seen in the non-induced lanes reflects a low level of QDE-1 expression. N, non-induced; I, induced.

MATERIALS AND METHODS

Neurospora crassa strains

The *N.crassa* wild-type strain 74-OR23A (FGSC No. 987) was obtained from the Fungal Genetics Stock Center, University of Kansas (KA, USA). The *qde-1*-overexpressing strain OQ1 was created by transforming a wild type with plasmid pMXY2:*qde-1* and purified by isolation of microconidia to obtain a homokaryon.

Neurospora crassa media and growth conditions

Strains were grown in Vogel's minimal medium for *Neurospora* (NMM) (15) plus benomyl (1 μg/ml) or hygromycin (0.2 mg/ml in slants and liquid media, or 0.3 mg/ml in solid media), as required. *Neurospora* strains transformed with *al-1* were grown for 48 h in liquid medium at 28°C in the dark, with constant shaking at 150 r.p.m. [for growth in induced conditions, liquid medium contained 0.5% sucrose, 1× Vogel's and 0.6% quinic acid (QA)]. Expression of *al-1* was induced by constant saturating light for 20 min at 10 W/m^2.

Plasmids

The *qde-1* overexpression plasmid pMXY2:*qde-1* was created by insertion of the *qde-1* gene into the SmaI site immediately downstream of the inducible QA dehydrogenase (*qa-2*) promoter in the pMXY2 vector that contains benomyl resistance as a selectable marker (16). Plasmid pCSN44 carrying the hygromycin resistance gene (17) was used in co-transformation with the pX16 plasmid carrying the *al-1* sequence (18) to induce silencing.

Transformation

Preparation of *N.crassa* spheroplasts and transformation with recombinant plasmids was performed as described by Vollmer and Yanofsky (19). Transformants were selected by growth on plates containing selectable markers, as required, and subsequently transferred to slants.

Southern analysis

Genomic *N.crassa* DNA was prepared as described in (20). Five micrograms of chromosomal DNA was digested with SmaI/HindIII and fractionated by electrophoresis on a 1% agarose gel. DNA was transferred onto Gene Screen Plus (NEN) filters by capillary blotting. Filters were hybridized according to the manufacturer's instructions at 65°C with a labelled 1.3-kb XbaI–ClaI fragment of the *al-1* gene, which is able to detect both endogenous and transgenic *al-1* sequences. Probes were ^{32}P-labelled using a Random Primed DNA Labelling Kit (Roche) according to the manufacturer's instructions.

Northern analysis

Total RNA was extracted from frozen mycelia. Five micrograms of RNA, quantified by spectrophotometric analysis, were run on a 1% agarose formaldehyde gel and blotted onto HybondN membranes (Amersham Corp). Membranes were hybridized with a 500-bp fragment of the *qde-1* gene amplified by PCR from wild-type DNA using Amplitaq DNA polymerase (Perkin Elmer) using forward primer 5′-GCTGGACACTTGATTGAG-3′ and reverse primer 5′-GTCATTGCGGTCACGAAC-3′, and labelled as described above. The *al-1* probe was same as that used for the Southern analysis. Quantitative analysis was carried out by electronic autoradiography (Packard Instant Imager).

Purification by microconidia

Purification was carried out as described previously (21).

Small RNA purification and hybridization

These were performed as described previously (4). Hybridization was carried out with a labelled XbaI–ClaI fragment of the *al-1* gene.

RESULTS

Overexpression of *qde-1* increases silencing efficiency

In *N.crassa*, the *albino* genes involved in the carotenoid biosynthetic pathway are used as a visual reporter system, since quelling of *albino-1* (*al-1*) encoding phytoene dehydrogenase confers an albino phenotype. The *N.crassa* strain OQ1 (carrying an inducible *qde-1* transgene) (Fig. 1) and a wild-type strain were co-transformed with plasmids pX16 (17), to trigger silencing of *al-1*, and pCSN44, conferring hygromycin resistance, as a marker of co-transformation (see Materials and Methods). Transformants grown in conditions of induced and non-induced *qde-1* overexpression may be divided into those that are 'non-silenced' (NS) or 'silenced' (S), irrespective of *qde-1* overexpression, and those that are 'inducibly silenced' (IS), exhibiting a silenced phenotype only when *qde-1* is overexpressed (Fig. 2). The percentage of transformed wild-type colonies with an albino phenotype due to silencing of *al-1* was 22%, while for OQ1 transformants the level of silencing increased to 92% in induced conditions, compared with 21% observed in non-induced conditions (Table 1). In order to verify that the high level of silencing was a general phenomenon, and not specific to *al-1*, OQ1 was transformed with *albino-2* (*al-2*) encoding phytoene synthase, which is also

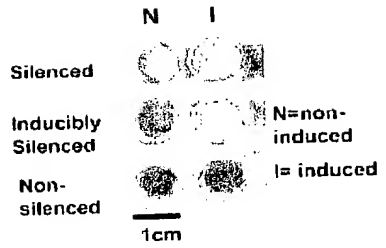

Figure 2. Effect of *qde-1* overexpression on the phenotype of *al-1* transformants. Phenotype of a non-silenced (orange) and a silenced (albino) strain, irrespective of *qde-1* overexpression, and an inducibly silenced strain where silencing (albino phenotype) was a result of *qde-1* overexpression induced by quinic acid.

involved in carotenogenesis. Similar levels of silencing of *al-2*, as indicated by an albino phenotype, were observed (Table 1).

The results above indicate that in some transgenic strains (S strains), the silencing signal is sufficient to induce substantial mRNA degradation, whereas in other transformants (IS) silencing only occurs when *qde-1* is overexpressed. Consistent with this hypothesis, we found that al-1 siRNA levels are increased in induced conditions, both in constitutively silenced (OQ1-S1) and inducibly silenced (OQ1-IS60) strains (Fig. 3). Characteristically, both constitutively and inducibly silenced strains show an accumulation of siRNAs in induced and non-induced conditions, but in the OQ1-IS60 strain the siRNA level, which is lower than in the OQ1-S1 strain, does not appear to be sufficient for mRNA degradation.

The increase in silencing frequency and the isolation of silencing-inducible transgenic strains strongly suggests that the overexpression of *qde-1* acts on a (pre-existing) silencing signal (present in almost all transgenic strains) by increasing the amount of dsRNA synthesized, consequently augmenting the level of siRNAs. It could be argued, however, that the increased silencing frequency is affected by uncontrollable parameters during transformation, altering the number of transgenic copies integrated, the site(s) of integration and/or organization. To overcome this objection we carried out a forced heterokaryon experiment where a non-silenced wild-type transformant containing the *al-1* transgene was forced with the *qde-1* overexpresssing strain (no copies of transgenic *al-1*). The resulting heterokaryon showed a silenced phenotype when QDE-1 was overexpressed by quinic acid. Instead, when the non-silenced wild-type *al-1* transformant was forced with a wild-type strain, no silencing was seen. These data suggest that QDE-1 is a 'limiting factor', since the level of transgenic RNA remained the same in both heterokaryons. In addition, these data support the notion that in a wild-type background a given transgenic locus is able to produce a silencing signal, but that this signal is not always sufficient to induce silencing.

Overexpression of *qde-1* reduced the number of copies required for silencing

Given that a non-silenced (NS) wild-type *al-1* with a few copies can be silenced by creating a heterokaryon with overexpressed *qde-1*, we asked if the number of transgenic copies required to elicit a silencing response is reduced when OQ1 is overexpressed. We therefore carried out a Southern analysis of *al-1* copy number in 52 OQ1 *al-1* transformants, including a wild-type strain as a control for the endogenous copy (giving a 3.1-kb band) (Fig. 4A and B). Tandem arrays of the *al-1* transgene revealed by a 5.5-kb band were present in S strains (OQ1-S1, -5 and -7), confirming previous data demonstrating that a high transgene copy number is required for efficient silencing. In comparison, the IS strains (OQ1-IS9, -10, -20, -26, -13 and -22) harboured fewer copies of the *al-1* transgene, even as few as one or two copies (OQ1-IS10 and -20), indicating that overexpressing *qde-1* compensates for the lower *al-1* copy number, i.e. the number of transgenes required to trigger silencing is reduced when QDE-1 is not limiting. Interestingly, 66% of the NS strains (8 out of 12 strains) contained no copies of *al-1*, indicating that a large portion of the 22% of NS OQ1 *al-1* transformants may have been transformed with pCSN44 only (conferring hygromycin resistance) and are therefore 'false negatives', suggesting that the actual level of silencing in conditions of *qde-1* overexpression is ~92% (see Table 1). As quelling is known to progressively revert phenotype back to wild type, which is associated with a loss of tandemly repeated copies over a prolonged culture time (12), and since we have IS strains containing one or two copies, we may expect the stability of the phenotype in overexpressed strains to be increased. We

Table 1. Effect of *qde-1* overexpression on the efficiency of silencing in *al-1* and *al-2* transformants in OQ1 and wild-type (WT) strains

Gene	Non-silenced (%)	Silencing in non-induced conditions (%)	Silencing in induced conditions (%)	Frequency of silencing excluding NS transformants with no *al-1* transgene (%)	Total numbers analysed (*n*)
OQ1 strain					1400
al-1	22	21	78	92	780
al-2	23	23	73	90	
Wild-type strain					500
al-1	78	22	22	NA	220
al-2	81	19	20	NA	

NS, non-silenced; NA, not available.
Colonies were either non-silenced (orange phenotype) or silenced (albino phenotype) irrespective of *qde-1* overexpression, or inducibly silenced (albino phenotype) due to *qde-1* overexpression induced by quinic acid. The frequency of silencing considers only those transformants that contain the *al-1* transgene and was calculated by analysing non-silenced transformants for copy number. Two-thirds (66%) of the NS strains (eight out of 12 strains analysed) contained no copies of *al-1* and are therefore 'false negatives', putting the actual level of silencing in conditions of *qde-1* overexpression at ~92%.

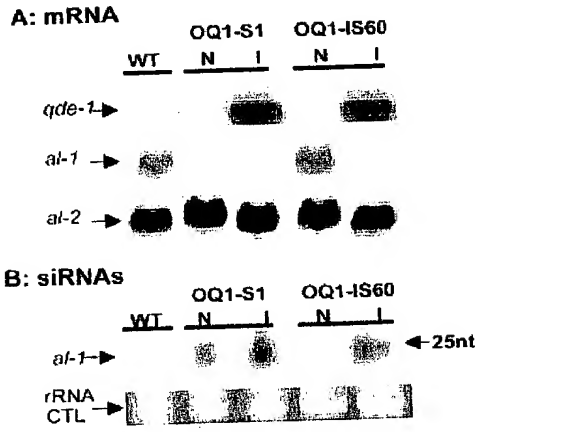

Figure 3. Northern analysis of mRNA and siRNA. (A) Northern analysis of *qde-1* and *al-1* mRNA in a wild-type (WT) control and two overexpressed strains in both non-induced (N) and induced (I) conditions. *al-1* mRNA was silenced in OQ1-S1, whereas for OQ1-IS60 it was only silenced when *qde-1* was overexpressed. Expression of the *al-2* gene was assessed for normalization purposes. (B) Northern analysis of *al-1* siRNAs in the same strains as in (A). As expected, no *al-1* siRNAs were detected in the wild type, whereas in OQ1-S1 and OQ1-IS60 an increase of *al-1* siRNA was detected under induced conditions. The ribosomal RNA is shown as a control for equal loading.

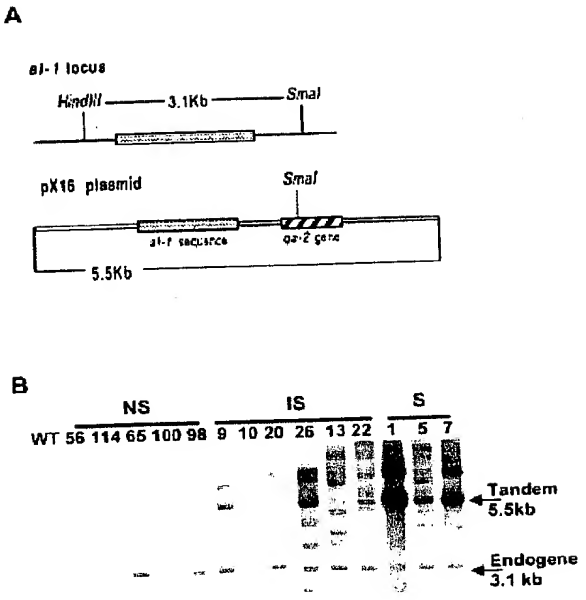

Figure 4. NS, non-silenced; IS, inducibility silenced; S, constitutely silenced. (A) Southern analysis of *al-1* copy number in transformed wild-type and OQ1 strains. Schematic representation of the *al-1* endogenous locus and of plasmid pX16 carrying the *al-1* transgene. (B) Southern analysis of *al-1* copy number in wild-type and OQ1 strains transformed with pX16. Eleven out of a total data set of 52 are shown. Lane 1: wild type (control); lanes 2–6: non-silenced OQ1 strains; lanes 7–12: inducibly-silenced OQ1 strains; lanes 13–15: silenced OQ1 strains. The 3.1-kb band corresponds to the endogenous *al-1* gene and the 5.5-kb band corresponds to ectopic *al-1* transgenes. The more intense 5.5-kb signal in silenced OQ1 strains denotes tandem arrays of transgenic *al-1* sequences, while diverse bands represent randomly integrated transgene copies. Non-silenced OQ1 transformants harbour only one, two or no *al-1* transgenes.

therefore compared the level of reversion between six OQ1 and five wild-type *al-1* transformants. All transformants were purified by isolation of microconidia to ensure that they were homokaryotic (21). Table 2 shows that wild-type *al-1* strains have a reversion rate of up to 28% in *al-1* transformants. Only one of the OQ1 strains (OQ1-IS14) showed reversion of a single colony out of 5700 descendents. The other five strains showed no reversion. To determine whether these strains still continued to lose copies of the transgene, we analysed 50 single colonies of OQ1-S1, OQ1-IS60 and WT-S3. Whilst OQ1-S1 and OQ1-IS60 showed no phenotypic reversion (Fig. 5A), Southern analysis revealed a loss of the *al-1* tandem array at a rate similar to that of wild-type strains, i.e. ~30% (Fig. 5B). Quantification of copy number using electronic autoradiography revealed that reversion of silencing occurred when less than six transgenic *al-1* copies remained in tandem, whereas in OQ1 strains, silencing was maintained even in the presence of only two copies. This confirms that there is a threshold number of copies required to elicit a silencing response, and that this number is reduced when *qde-1* is overexpressed.

Table 2. Phenotypic reversion of *al-1* transformants of OQ1 and wild-type strains

Transformant	Number analysed	No. of reversions (%)
1. OQ1-S7	764	0 (0)
2. OQ1-S1	1925	0 (0)
3. OQ1-IS13	3160	0 (0)
4. OQ1-IS14	5700	1 (0.017)
5. OQ1-IS60	3570	0 (0)
6. OQ1-IS72	3934	0 (0)
7. WT-S1	1600	1 (0.06)
8. WT-S2	3500	100 (2.86)
9. WT-S3	1880	540 (28)
10. WT-S12	1580	0 (0)
11. WT-S207	1100	70 (6.3)

DISCUSSION

RNA-dependent RNA polymerases are key components of the RNAi machinery. Two different roles have been postulated for these enzymes: first, RdRPs may be involved in converting aberrant RNA molecules produced from silencer loci into dsRNA, or secondly, use siRNAs as primers in the conversion of the target ssRNA into dsRNA, leading to both the degradation of the target RNA by dicer and the accumulation of secondary siRNAs, thus increasing the strength of silencing. Interestingly, we have found that QDE-1 was no longer required upon the direct expression of dsRNA (22). Likewise, in plants, the RdRP SGS2 is not required when PTGS is induced by an inverted repeat (23). These results suggest that the main role of RdRP in transgene-induced gene silencing in *Neurospora* is the conversion of transgenic RNA into dsRNA. In line with this result, in this work we found that QDE-1

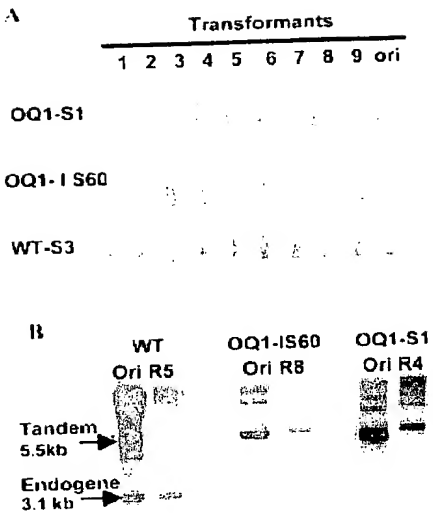

Figure 5. Reversion of silencing in OQ1 and wild-type *al-1* transformants. (**A**) Phenotype of the original culture ('ori') with respect to nine single colonies of a silenced OQ1 transformant (OQ1-S1), an inducibly silenced OQ1 transformant (OQ1-IS60) and a silenced wild-type *al-1* transformant (WT-S3). (**B**) Southern analysis of *al-1* copy number in the original culture (ORI) as compared with revertant R5 of WT-S3, R8 of OQ1-IS60 and R4 of OQ1-S1. Around 30% of the descendants showed a characteristic loss of transgenic copies.

overexpression resulted in an increase in the production of siRNAs. QDE-1 is thought to recognize transgenic RNAs due to some intrinsic 'aberrancy' rendering them substrates for RdRPs. Previous reports have suggested that in fungi and plants, a prerequisite for efficient PTGS is the presence of multiple tandemly inserted copies of a transgene and that the abRNA that results from the transcription of such a multi-copy transgenic locus is the trigger of PTGS (11,12). Such tandems are also proposed to be able to maintain silencing through primed polymerization by RdRP, regenerating dsRNA, where single copies would be exhausted by the sequential use of downstream primers (24). We found that the overexpression of the *qde-1* gene leads to a dramatic increase in the frequency of transgenic strains showing silencing. In fact, we observed that the silencing frequency of *al-1* is ~92% when QDE-1 is overexpressed, compared with 22% observed in a wild-type background, indicating that almost all transgenic strains are able to support silencing. Importantly, we found that one or two copies can elicit a silencing response when *qde-1* is overexpressed, suggesting that rather than a tandem repeat being essential for triggering silencing, every transgenic/repetitive locus possesses the ability to activate silencing. This is in line with early experiments in fungi and plants, which showed that the presence of a tandem repeat *per se* is not sufficient to elicit silencing (13,14). The fact that only a limited number of transgenic strains (those with the highest number of transgenes) show silencing in the presence of a wild-type level of QDE-1 suggests that silencing in *Neurospora* is only activated if a given threshold of transgenic/repetitive copies is reached. In reality, such a threshold may reflect 'quantitative effects', i.e. silencing may be a gradient from weak to strong, rather than on or off, with the overexpression of *qde-1* allowing detection of the silenced phenotype where less transgenic copies are present. Visual inspection of conidial colour in our system does not allow these two models to be differentiated.

Such high levels of silencing are reminiscent of a mutant phenotype seen in *Caenorhabditis elegans*, in which *rrf-3* mutants show hypersensitivity to RNAi (25). The authors suggest that the RRF-3 protein competes with the other required RdRPs RRF-1 and EGO-1 for components or intermediates in the RNAi pathway. We could envision a similar situation here, i.e. that QDE-1 outcompetes the other RdRPs for its RNA substrate, resulting in a higher silencing efficiency. However, mutants in the other two RdRP paralogs of *Neurospora sad-1* and *RdRP-3* show normal (wild type) silencing efficiencies (our unpublished data), indicating that such a mechanism is not at work in *Neurospora*.

Quelling is known to revert by excision of tandemly arranged copies during vegetative growth, therefore suggesting that a threshold number of transgenic copies is required not only to activate, but also to maintain silencing. The fact that we found that the phenotypic stability of silencing is strongly increased when *qde-1* is overexpressed, even though copies are lost at the same rate as in wild type, indicates that high levels of QDE-1 allow the maintenance of silencing even when the number of transgenic copies are reduced by increasing the production of dsRNA and in turn siRNA molecules. This is further demonstrated by the forced heterokaryon experiment, where the same amount of transgenic RNA produced by *al-1* transformed nuclei is able to support silencing only in the presence of the nuclei that overexpress QDE-1. Together our results suggest that in *Neurospora*, silencing activation and maintenance appear to rely on both the cellular amount of QDE-1 and the amount of transgenic copies producing RNA molecules that act as a substrate for the RdRP, implicating QDE-1 as a rate-limiting factor in PTGS.

ACKNOWLEDGEMENTS

We would like to thank Annette Pickford for critical reading, suggestions and revision of the manuscript, and the Whitehead Institute for providing access to the *Neurospora* genome database. This work was supported by grants from the European Community (QLK3-CT-2000-00078), the Instituto Pasteur Fondazione Cenci Bolognetti, FIRB-MIUR 2001 (RBNEO15MPB_001/RBNE01KXC9_006) and CNR 2003 (Progetto Strategico MIUR: legge 449/97).

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